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Editorial Office

Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku, Tokyo 112-0003, Japan
E-mail: office@biosciencetrends.com

BioScience Trends

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

E-mail: office@biosciencetrends.com
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Original Article

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Depletion of circ-BIRC6, a circular RNA, suppresses non-small cell lung cancer progression by targeting miR-4491

Zhu Jin, Baoan Gao*, Yuan Gong, Li Guan

Institute of Respiratory Disease, China Three Gorges University, Yichang Central People's Hospital, Yichang, Hubei, China.

SUMMARY Circular RNAs (circRNAs) are non-coding RNAs molecules consisting of a covalently closed continuous loop which have no 5'-3' polarity and contain no polyA tail. Accumulating evidence demonstrates that circRNAs are involved in the initiation and progression of human malignancies. In this study, we explored the expression profile and regulatory role of circ-baculoviral IAP repeat-containing 6 (circ-BIRC6), a circular RNA, in malignant behaviors in non-small cell lung cancer (NSCLC). Expression levels of circ-BIRC6 and miR-4491 were examined in NSCLC patient samples and cell lines using quantitative real time PCR (qRT-PCR). *In vitro* roles of circ-BIRC6 knockdown on cell viability, colony formation, and apoptosis were assessed using the CCK-8, colony formation assay, and flow cytometry, respectively. The interactions between circ-BIRC6 and miR-449 were assessed using luciferase reporter and qRT-PCR assays. The *in vivo* role of circ-BIRC6 knockdown on tumor growth and apoptosis was evaluated in a xenograft mouse model of NSCLC. We found that expression levels of circ-BIRC6 in NSCLC patient samples and cell lines were elevated. Small interfering RNA (siRNA)-mediated circ-BIRC6 knockdown suppressed cell proliferation, colony formation, migration and invasion, and promoted apoptosis in NCI-H460 and A549 cells. In addition, miR-4491 was identified as a tumor-suppressor miRNA in NSCLC and circ-BIRC6 functions as a molecular sponge for miR-4491. Furthermore, circ-BIRC6 knockdown suppressed Wnt2B/β-catenin pathway. *In vivo* assay showed that depletion of circ-BIRC6 suppressed tumor growth, enhanced apoptosis, and decreased miR-4491 levels in a mouse xenograft model. These findings demonstrate that circ-BIRC6 functions as a critical regulator of proliferation and apoptosis *via* binding to and negatively regulating miR-4491, suggesting that circ-BIRC6 might be a potential target for treatment of NSCLC.

Keywords non-small cell lung cancer, Circular RNA, Circ-BIRC6, MiR-4491

1. Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths. Although great improvement has been achieved in cancer therapy, approximately 40% of NSCLC cases have advanced-stage disease with a five-year survival rate of approximately 2% (*1,2*). Therefore, further understanding of the molecular mechanisms and development of novel therapies with enhanced specificity are immediately needed for the management of NSCLC.

Circular RNAs (circRNAs), a novel type of RNA, are a special group of non-coding endogenous RNAs molecules, consisting of at least a few hundred nucleotides (*3*). According to previous studies, most circRNAs are noncoding RNAs while only a few protein-coding circRNAs have been reported (*4*). Accumulating evidence supports the diverse roles of circRNAs in cellular behavior, including growth, apoptosis, migration, and differentiation. In addition, circRNAs play critical roles in the initiation and progression of human malignancies (*5-7*). Particularly, circ-BIRC6 (ID hsa_circ_0003288) is located on chromosome 2 (32703702–32718734) and is generated by back-splicing of the *BIRC6* transcript (NM_016252). It has been reported that levels of circ-BIRC6 are upregulated in hepatocellular carcinoma (HCC), and depletion of circ-BIRC6 could suppress cell growth, migration, and invasion, representing a potential prognostic factor and therapeutic target for HCC (*8*). As is well acknowledged, circRNAs regulate gene expression at the transcriptional or post-transcriptional level by binding to microRNAs (miRNAs) or other molecules (*9,10*). Mechanically, circ-BIRC6 is reported to function as an oncogenic gene *via* sponging miR-3918 in HCC (*8*). Although a close relationship between circRNAs and NSCLC has been reported, the

specific mechanisms by which circRNAs may regulate carcinogenesis and progression of NSCLC remains largely unknown (*11*). Herein, our study explored the expression pattern of circ-BIRC6 and its effects on the malignant behaviors in NSCLC.

2. Materials and Methods

2.1. Patient samples

NSCLC tissues and paired adjacent noncancerous lung tissues (n = 30) were obtained in the Yichang Central People's Hospital. NSCLC patients were pathologically diagnosed and received no chemotherapy nor radiotherapy before tissue sampling. The samples were immediately frozen and stored in liquid nitrogen. This study was approved by the Ethics Committee of Yichang Central People's Hospital and written informed consent was obtained from all participants.

2.2. Cell culture

Human lung cancer cell lines, including NCI-H460 and A549, were obtained from the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (CAS, Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 μg/mL streptomycin, and 100 μg/mL penicillin (Hyclone, Logan, UT, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Cell viability assay

CCK-8 assay was performed for determining cell viability, using the CCK-8 assay kit according to the manufacturer's protocol. In brief, cells were seeded into 96-well plates and transfected with negative control construct or target gene-manipulated construct. The cell viability in each well was determined by adding 10 μLof CCK-8 solution. After further incubation at 37°C for 2 h, absorbance was measured using an ELISA reader at a wavelength of 450 nm.

2.4. Cell transfection

Cells were plated in 6-well plates and transfected with siRNA for circ-BIRC6 and negative control (si-NC), according to the manufacturer's instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced six hours after transfection. The interference sequences were circ-BIRC6 siRNA, 5'- CUGAAAGGUUCUUGCACGCAU-3'. Scramble siRNA (5'- GACUUUCGUUCUUGCACGCAU-3') was used as a negative control.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using Trizol reagent. The isolated RNA was reverse transcribed into cDNA, which was used as a template to perform qRT-PCR on an ABI 7900 system. The transcript levels of indicated genes were determined using the 2-ΔΔCt. Primers used in the study were as follows: circ-BIRC6, 5'-TGAAAGGTTCTTGCACGCAT-3' and 5'-GCTGGGGTTCGTTCACAATC-3'; β-actin, 5'-CACAGAGCCTCGCCTTTGCC-3'; 5'-ACCCATGCCCACCATCACG-3'; miR-4491, 5'-GTTCTGCGTCCCAGGCAGT-3'; 5'-GCTAACGGCTGATGTGTCCA-3'; U6, 5'-CGAGCACAGAATCGCTTCA-3'; 5'-CTCGCTTCGGCAGCACATAT-3'. U6 levels were used to normalize miR-4491 expression. β-actin was endogenous control for circ-BIRC6. All reactions were performed in triplicates.

2.6. Colony formation assay

Tumor cells were plated at 1×10^6 cells/plate in a 100-mm culture dish one day before transfection. The next day, cells were transfected with negative control construct or target gene-manipulated construct. The surviving colonies were stained with crystal violet and visible colonies were counted using an automated colony-counting system (Media Cybernetics, Inc., Bethesda, MD, USA).

2.7. Flow cytometry

Cell apoptosis was analyzed using annexin V/PI apoptosis kit (Invitrogen, USA) according to the manufacturer's instruction. In brief, cells were seeded in a 6-well plate and transfected with the siRNA negative control or siRNA against circ-BIRC6. The cells from each group were washed with ice-cold PBS, resuspended in 100 μL binding buffer, and stained with annexin V-FITC and PI. Apoptosis of samples were then analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

2.8. Terminal deoxynucleotidyl transferase d-UTP nick end labelling (TUNEL) staining

Cell apoptosis was analyzed by TUNEL assay using an in-situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. In brief, cells were fixed in 4% paraformaldehyde, incubated in 3% H_2O_2, permeabilized with 0.5% Triton X-100, and then incubated in the TUNEL reaction mixture. Sections were rinsed, stained with DAPI, and observed under a fluorescence microscopy.

2.9. Transwell assays

Transwell assay was used to determine the invasion and migration of cells. For tumor invasion assay, Transwell

chambers (Costar, Cambridge, MA, USA) were coated with Matrigel on the upper surface. Cells were suspended in serum-free medium in the upper chamber, and lower chamber was added with medium containing 10% FBS. Forty-eight hours later, cells in upper chambers were removed and stained to detect the invaded cells. The migration assay was performed with the same procedure in Transwell chambers without Matrigel. The numbers of migrated and invaded cells were calculated.

2.10. Luciferase reporter assay

Cells were co-transfected with luciferase reporter plasmids carrying circ-BIRC6 wt or circ-BIRC6 mut, and miR-4491 mimics or miR-NC plasmids using Lipofectamine 2000. Then, firefly and Renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay System Kit (Promega) to analyze the interaction between circ-BIRC6 and miR-4491.

2.11. Western blotting

Cultured cells were lysed in cell lysis buffer and protein samples were separated by SDS-PAGE. After separation, proteins were transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA) followed by incubation with primary antibodies anti-bax (dilution 1:2,000, Santa Cruz, USA), Wnt2B (dilution 1:2,000, Santa Cruz, USA), β-catenin (dilution 1:2,000, Santa Cruz, USA) and anti-bcl-2 (dilution 1:2,000, Santa Cruz, USA), overnight at 4ºC. After washing in PBS, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:5,000) at room temperature for 1 h. The membranes were then washed in PBS for three times, and protein bands were detected using the enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA). Protein bands were captured using the Leica Image Processing system and processed using Image J software (National Institutes of Health, USA), and their intensity was normalized to that of the band for β-actin (1:2,000).

2.12. Xenograft model

Female BALB/c mice (4-6 weeks old) were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). All procedures for animal studies were approved by the ethics committee of Yichang Central People's Hospital. Wild-type or circ-BIRC6-knockdown tumor cells were injected subcutaneously into nude mice (n = 5 per group). Subsequently, mice in each group were intraperitoneally injected with cisplatin (20 mg/kg body weight) and tumor formation was observed. Tumors were measured once a week for five weeks followed by determination of tumor volume and tumor weight. For tumor volume, the following formula was used: $V = \pi/6 \times L \times W2$, where V = volume (mm3), L = length (mm), and W = width (mm).

2.13. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences between two groups were compared with Student test, while differences between multiple groups were compared using ANOVA followed by Tukey tests. Results with a P value < 0.05 were considered statistically significant.

3. Results

3.1. circ-BIRC6 is upregulated in NSCLC tissues and cell lines

Expression profile of circ-BIRC6 in NSCLC patient samples and cell lines was determined using quantitative real time PCR (qRT-PCR). Data showed that the expression of circ-BIRC6 was significantly upregulated in NSCLC tissues compared to the adjacent normal tissues (Figure 1A). Additionally, levels of circ-BIRC6 in two NSCLC cell lines, including NCI-H460 and A549,

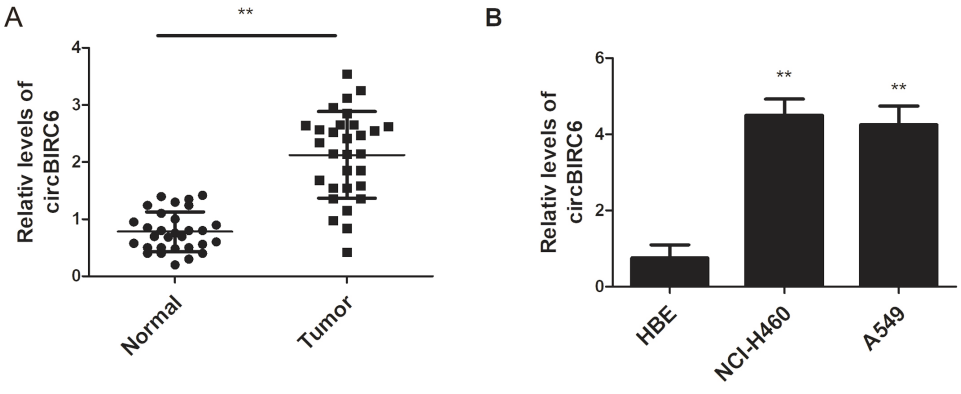

Figure 1. circ-BIRC6 is upregulated in NSCLC tissues and cell lines. Relative expression of circ-BIRC6 in 30 pairs of NSCLC and normal tissue was determined by qRT-PCR **(A)**. In addition, expression of circ-BIRC6 was detected in NCI-H460, A549 and human bronchial epithelial cells **(B)**. Results are expressed as mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$.

and the human bronchial epithelial cell line (HBE), were detected. Consistently, we found that circ-BIRC6 was overexpressed in NCI-H460 and A549 cells compared to HBE cells (Figure 1B). These results imply a possible oncogenic role of circ-BIRC6 in the progression of NSCLC cells.

3.2. circ-BIRC6 depletion suppresses malignant behaviors of NSCLC cells

To explore the potential oncogenic roles of circ-BIRC6 *in vitro*, NCI-H460 and A549 cells transfected with designed siRNA specifically targeting circ-BIRC6. Transfection with this siRNA effectively silenced circ-BIRC6 levels in NCI-H460 and A549 cells (Figure 2A) but had no effect on linear BIRC6 mRNA levels (Figure 2B). Consequently, siRNA-mediated knockdown of circ-BIRC6 remarkably inhibited cell proliferation and colony formation (Figure 2C and D). In addition, we found that depletion of circ-BIRC6 significantly induced apoptosis compared to the scramble control in NCI-H460 and A549 cells, as determined by flow cytometry (Figure 3A and B). On the molecular level, western blot revealed that knockdown of circ-BIRC6 dramatically promoted the expression of bax and suppressed bcl-2 in NCI-H460 and A549 cells (Figure 3C). Furthermore, transwell analysis showed that downregulation of circ-BIRC6 inhibited migration and invasion of NCI-H460 and A549 cells (Figure 3D) Collectively, these findings demonstrated that knockdown of circ-BIRC6 could exert the anti-tumor properties in NSCLC cells.

3.3. circ-BIRC6 binds directly to miR-4491 in NSCLC cells

To elucidate the underlying mechanism by which circ-BIRC6 manipulates NSCLC progression, we screened potential targets of circ-BIRC6 using online software Circular RNA Interactome (*http://regrna2.mbc.nctu.edu.tw/*) and identified that miR-4491 as a candidate target (Figure 4A). Real-time PCR results showed that circ-BIRC6 depletion led to an obvious increase of miR-4491 in NCI-H460 and A549 cells (Figure 4B). To validate the hypothesis that circ-BIRC6 serves as a miRNA sponge, we generated wild type (wt) circ-BIRC6 luciferase plasmids containing potential miR-4491 binding sites, as well as mutant variants of binding site. Following co-transfection, the luciferase activity was lower in the circ-BIRC6 wt+miR-4491 group than in the si-NC group, while was comparable in circ-BIRC6-mut+miR-4491 group and the si-NC group, in both NCI-H460 and A549 cells (Figure 4C). Taken together, these results demonstrated that circ-BIRC6 can act as a molecular sponge for miR-4491 in NSCLC cells.

3.4. miR-4491 overexpression suppresses cell proliferation in NSCLC

In addition, we explored whether cell viability was affected by miR-4491 overexpression. Firstly, we detected levels of miR-4491 in NSCLC samples and cell lines, as determined using qRT-PCR. Data showed

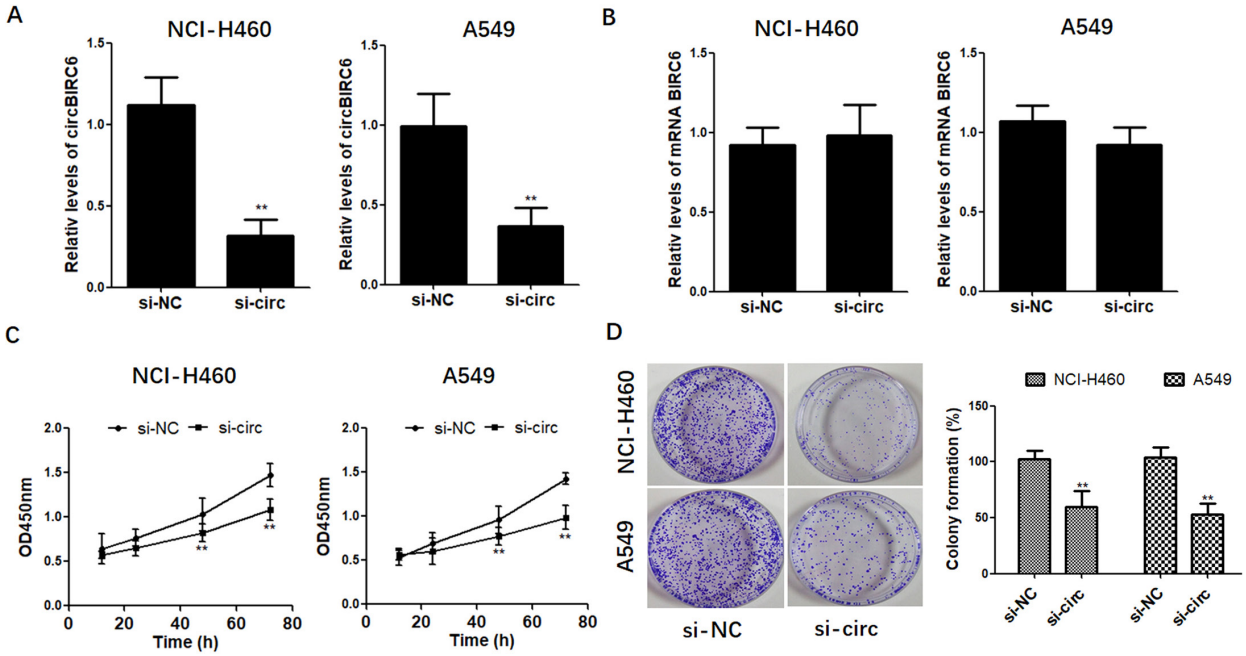

Figure 2. circ-BIRC6 depletion suppresses cell viability and colony formation in NSCLC cells. NCI-H460 and A549 cells were transfected with circ-BIRC6 siRNA or negative control. Levels of circ-BIRC6 and linear BIRC6 mRNA in NCI-H460 and A549 cells were determined using qRT-PCR **(A** and **B)**. In addition, cell proliferation and colony formation of NCI-H460 and A549 cells transfected with circ-BIRC6 siRNA were detected by CCK-8 assay **(C)** and colony formation assay **(D)**, respectively. Results are expressed as mean ± SD, $^{**}P < 0.01$.

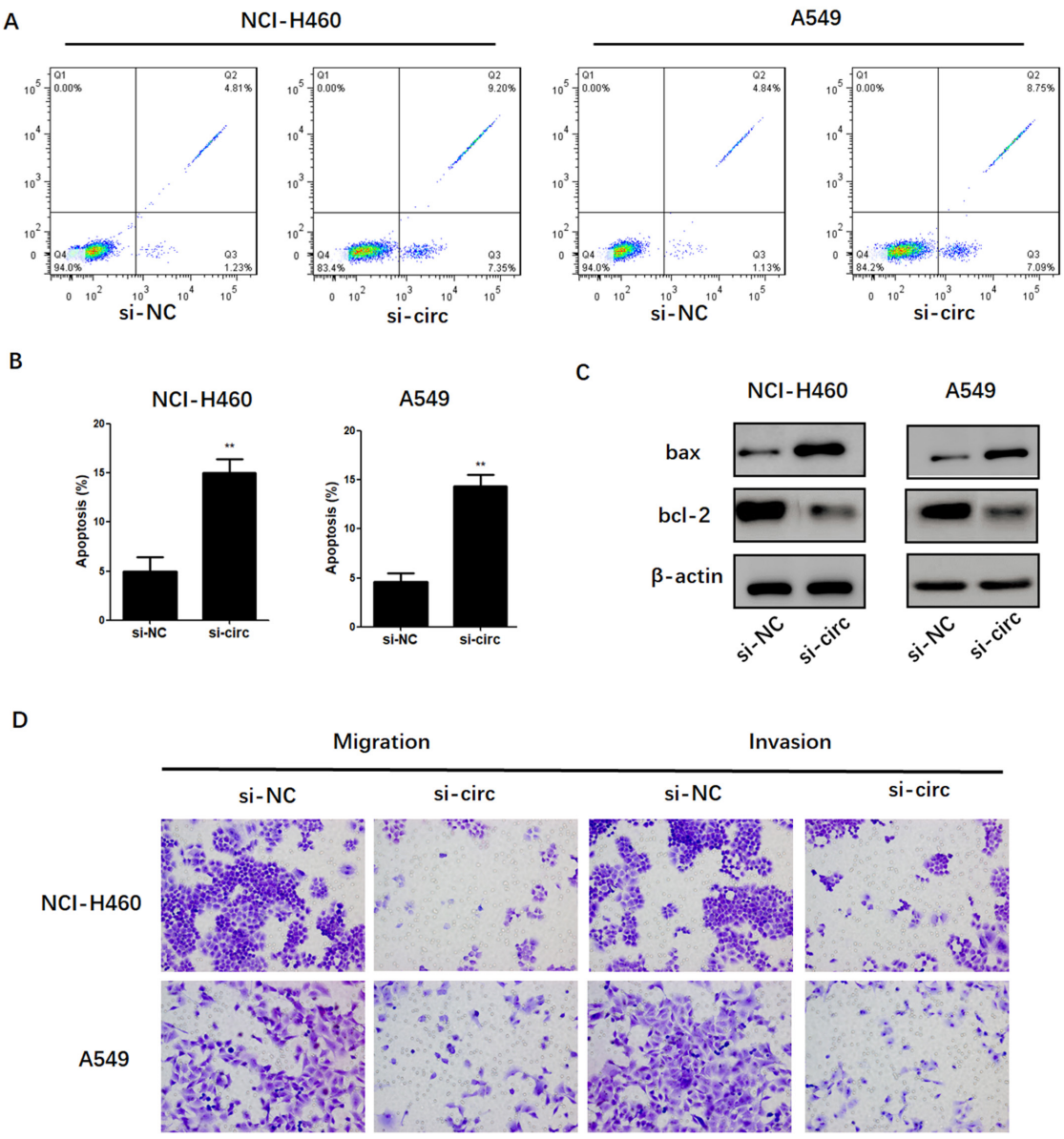

Figure 3. circ-BIRC6 depletion enhances apoptosis in NSCLC cells. NCI-H460 and A549 cells were transfected with circ-BIRC6 siRNA or negative control. Levels of apoptosis rates in NCI-H460 and A549 cells were determined using flow cytometry **(A** and **B)**. In addition, apoptosis-related proteins including bax and bcl-2 in NCI-H460 and A549 cells transfected with circ-BIRC6 siRNA were detected by western blot **(C)**. **(D)** Transwell analysis was conducted to measure the migration and invasion in NCI-H460 and A549 cells transfected with circ-BIRC6 siRNA. Results are expressed as mean ± SD, $^{**}P < 0.01$.

that miR-4491 was significantly downregulated in NSCLC tissues compared to the adjacent normal tissues (Figure 5A). Consistently, levels of miR-4491 were also decreased in NCI-H460 and A549 cells compared to HBE cells (Figure 5B). Strikingly, CCK-8 assay indicated that NCI-H460 and A549 cells ectopically expressed miR-4491 showed a slower growth rate, which were reversed after co-transfection with circ-BIRC6 (Figure 5C and D). Furthermore, miR-4491 was predicted to target Wnt2B (Figure 5E). Indeed, downregulation of circ-BIRC6 inhibited the expression of Wnt2B and β-catenin in NCI-H460 and A549 cells (Figure 5F). Taken together, these data suggested that miR-4491 exerted a tumor suppressor role *via* regulating Wnt2B/β-catenin signaling pathway in NSCLC cells.

3.5. *In vivo* effect of circ-BIRC6 knockdown on tumor growth

To explore the *in vivo* effect of circ-BIRC6-knockdown on tumor growth, NCI-H460 cells transfected with circ-BIRC6 siRNA or negative control, were injected into the flank of BABL/c nude mice. There was a significant reduction in tumor volume in mice injected with circ-BIRC6-knockdown cells compared to that in mice

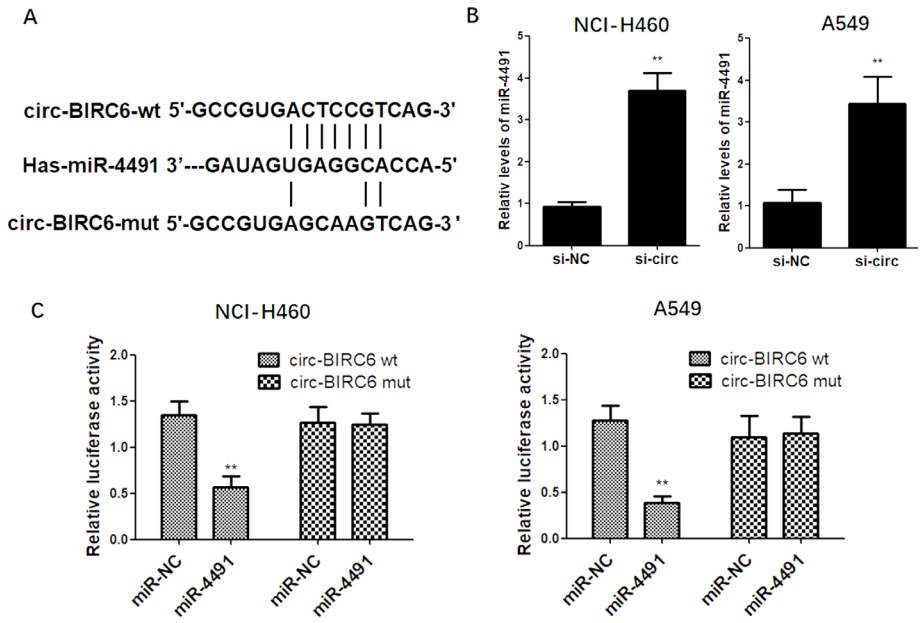

Figure 4. circ-BIRC6 binds directly to miR-4491 in NSCLC cells. NCI-H460 and A549 cells were transfected with circ-BIRC6 siRNA or negative control. Bioinformatics analysis of potential circ-BIRC6/miR-4491 interactions **(A)**. Then, expression of miR-4491 was detected in NCI-H460 and A549 cells transfected with circ-BIRC6 siRNA **(B)**. A dual luciferase reporter plasmid containing circ-BIRC6-wt or circ-BIRC6-mut was co-transfected into NCI-H460 and A549 cells along with miR-4491 mimics or miR-NC, and luciferase activities were determined **(C)**. Results are expressed as mean ± SD, $^{**}P < 0.01$.

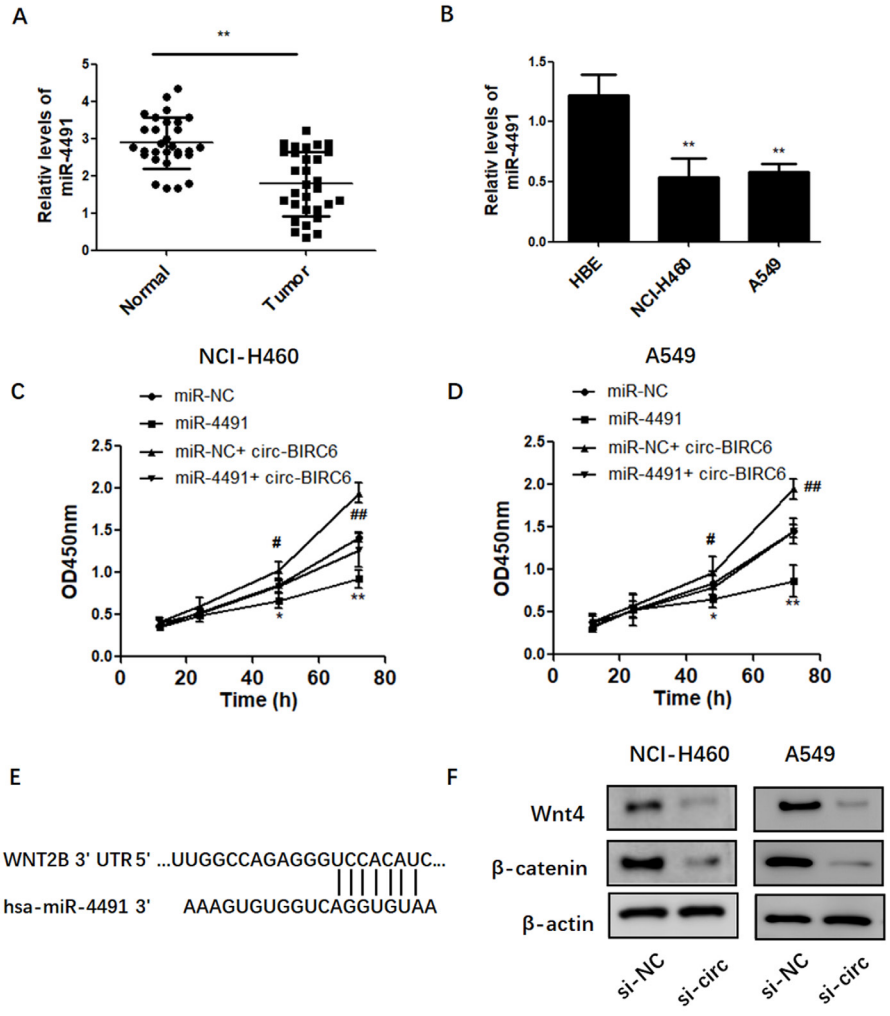

Figure 5. miR-4491 overexpression suppresses cell proliferation in NSCLC. Relative expression of miR-4491 in 30 pairs of NSCLC and normal tissue was determined by qRT-PCR **(A)**. $^{**}P < 0.01$, compared to normal. In addition, expression of miR-4491 was detected in NCI-H460, A549 and human bronchial epithelial cells **(B)**. Results are expressed as mean ± SD, $^{**}P < 0.01$, compared to HBE cells. In addition, CCK-8 assay was performed to determine the cell proliferation of NCI-H460 **(C)** and A549 **(D)** cells transfected with miR-NC, miR-4491 mimics, circ-BIRC6 alone miR-4491 mimics combined with circ-BIRC6. **(E)** Bioinformatics analysis of potential miR-4491/Wnt2B interactions. **(F)** Expression of Wnt2B and β-catenin in NCI-H460 and A549 cells transfected with circ-BIRC6 siRNA. Results are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to miR-NC.

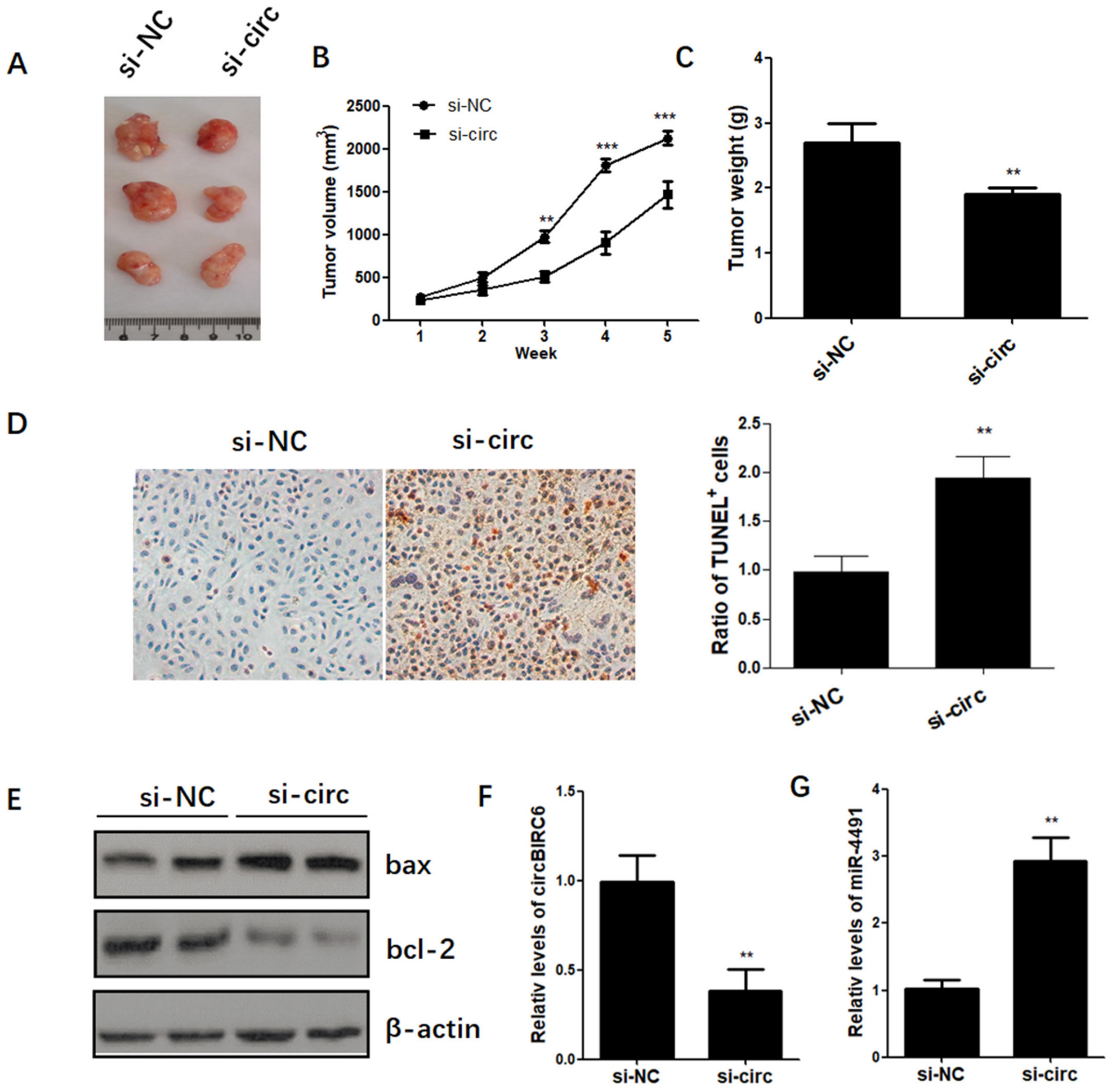

Figure 6. circ-BIRC6 depletion inhibits tumor growth *in vivo*. NCI-H460 cells were transfected with circ-BIRC6 siRNA or negative control, and injected subcutaneously into the flank of nude mice. Representative images of resected tumors **(A)**. Tumor volume and tumor weight were measured **(B** and **C)**. A TUNEL assay was performed to analyze the induction of apoptosis by circ-BIRC6 knockdown **(D)**. In addition, apoptosis-related proteins including bax and bcl-2 in resected tumors were detected by western blot **(E)**. Relative expression of circ-BIRC6 **(F)** and miR-4491 **(G)** in tumors. Results are expressed as mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$.

injected with control cells (Figure 6A-B). In addition, circ-BIRC6 depletion significantly decreased the tumor weight compared to that in control mice (Figure 6C). It was demonstrated by TUNEL assay that apoptosis rate in tumor cells was increased after depletion of circ-BIRC6 (Figure 6D). Consistently, western blot revealed that knockdown of circ-BIRC6 dramatically promoted the expression of bax and suppressed bcl-2 (Figure 6E). In addition, Moreover, expression of circ-BIRC6 was decreased while miR-4491 was elevated, in circ-BIRC6 siRNA-derived tumor tissues (Figure 6F and G). Collectively, these data indicate that depletion of circ-BIRC6 could exert a tumor suppressive role in NSCLC *in vivo*.

4. Discussion

NSCLC is the most common malignant tumor, ranking first among the reasons for cancer-related deaths worldwide. Despite therapeutic advances, the prognosis of patients with NSCLC is not satisfactory. Recent studies have demonstrated that circRNAs play critical roles in the pathogenesis and progression of malignancies, representing novel biomarkers and therapeutic targets in the management of NSCLC (*12,13*).For instance, hsa_circRNA_102958 is significantly in gastric cancer and correlated with TNM stage, representing a novel diagnostic marker for gastric cancer (*14*). Autophagy-associated circRNA circCDYL promotes breast cancer

progression *via* the miR-1275-ATG7/ULK1 axis (*15*), while circHIPK3 sponges miR-124-3p to promote proliferation and metastasis of lung cancer both *in vitro* and *in vivo* (*16*). Recently, Yang *et al.* identified high expression of circ-BIRC6 in NSCLC patients and found that it functioned as an oncogenic factor to promote HCC progression. Mechanistically, circ-BIRC6 promoted HCC cell proliferation, migration, and invasion *via* sponging miRNA-3918/bcl-2 axis (*8*). Additionally, RNA-sequencing revealed that 106 differentially expressed circRNAs, including 61 upregulated circRNAs and 45 downregulated circRNAs, in NSCLC cells treated with a small molecule inhibitor XAV939 (*11*). Herein, we explored the expression pattern of circ-BIRC6 in lung cancers and the underlying mechanisms in the pathogenesis of NSCLC. Our results detected elevated expression of circ-BIRC6 in patient samples with NSCLC and cell lines, suggesting a possible link between circ-BIRC6 and tumorigenesis in NSCLC. In addition, circ-BIRC6 knockdown decreased cell proliferation, colony formation, and induced apoptosis in NSCLC cells. The *in vivo* experiments also confirmed the suppressive effects of circ-BIRC6 knockdown on tumor growth.

Amounting evidences have demonstrated that miRNAs play an important role in the initiation and progression of colorectal (*17*), lung (*18*), and cervical (*19*) cancers. circRNAs contribute to initiation and progression of human malignancies by acting as miRNA sponges to regulate gene and protein expression (*20*). Recently, Chen *et al.* have shown that circRNA hsa_circ_100395 serves as a sponge for miR-1228 to inhibit progression and metastasis of lung cancer (*21*), while circTP63 functions as a ceRNA to promote lung squamous cell carcinoma progression by sponging miR-873-3p/FOXM1 axis (*22*). Herein, we identified downregulated expression of miR-4491 in NSCLC samples and cell lines; overexpression of miR-4491 exerted a tumor suppressor role in NSCLC cells. Furthermore, we identified that circ-BIRC6 functions as a molecular sponge for miR-4491. Both *in vitro* and *in vivo* assays suggested that circ-BIRC6 negatively regulated the expression of miR-4491, suggesting that circ-BIRC6/miR-4491 interaction mediates the progression of NSCLC. Particularly, we identified that Wnt2B/β-catenin signaling pathway was involved in circ-BIRC6-miR-4491-axis. Aberrant activation of the Wnt2B/β-catenin pathway plays a critical role in tumor initiation, progression, and metastasis of lung cancer (*23-25*). Our study found that downregulation of circ-BIRC6 inhibited the expression of Wnt2B and β-catenin in NCI-H460 and A549 cells.

In conclusion, circ-BIRC6 functions as a critical regulator of growth and apoptosis in NSCLC cells *via* sponging miR-4491. Our study suggests that circ-BIRC6 might be a novel molecular target for the management of NSCLC.

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Conflict of Interest: The authors have no conflict of interest to disclose.

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**Address correspondence to:*
Baoan Gao, Institute of Respiratory Disease, China Three Gorges University, Yichang Central People's Hospital, Yichang 443002, Hubei, China.
E-mail: jimama39@126.com

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Original Article

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Efficacy of early hydroxychloroquine treatment in preventing COVID-19 pneumonia aggravation, the experience from Shanghai, China

Yi Su[1,§], Yun Ling[2,§], Yuyan Ma[1,§], Lili Tao[3], Qing Miao[1], Qingfeng Shi[4], Jue Pan[1], Hongzhou Lu[2,*], Bijie Hu[1,*]

[1] Department of Infectious Diseases, Zhongshan Hospital Fudan University, Shanghai, China;
[2] Department of Infectious Diseases, Shanghai Public Health Clinical Center, China;
[3] UT Southwestern Medical Center, Department of Immunology, Texas, USA;
[4] Department of Infectious Control, Zhongshan Hospital Fudan University, Shanghai, China.

SUMMARY The aim of this study is to assess the efficacy of multiple treatments, especially hydroxychloroquine, used in different disease stages of coronavirus disease 2019 (COVID-19). All consecutive patients with COVID-19 admitted to Shanghai Public Health Clinical Center (Shanghai, China) between January 20, 2020, and April 30, 2020, were enrolled, and their clinical data were retrospectively collected. Binary logistic regression was used to screen the factors associated with disease aggravation, and multivariable analyses with the Cox proportional hazards model were used to estimate the effects of prognostic factors on the improvement time and PCR conversion days in throat swabs and stool swabs. A total of 616 patients, including 50 (8.11%) severe and 18 (2.92%) critical patients, were enrolled in our retrospective cohort study. The early use of hydroxychloroquine was a protective factor associated with disease aggravation (95% CI: 0.040-0.575, $p = 0.006$). Clinical improvement by 20 days was significantly different between patients with hydroxychloroquine used early and those with hydroxychloroquine not used ($p = 0.016$, 95% CI: 1.052-1.647). The median time to clinical improvement was 6 days in the hydroxychloroquine used early group, compared with 9 days in the without hydroxychloroquine used group and 8 days in the with hydroxychloroquine not used early group ($p < 0.001$). Hydroxychloroquine used early was associated with earlier PCR conversion in both throat swabs (HR = 1.558, $p = 0.001$) and stool swabs (HR = 1.400, $p = 0.028$). The use of hydroxychloroquine at an early stage is a potential therapeutic strategy for treating patients before irreversible severe respiratory complications occur. The early use of hydroxychloroquine decreased the improvement time and the duration of COVID-19 detection in throat and stool swabs.

Keywords clinical management, treatment, hydroxychloroquine, COVID-19, SARS-CoV-2, coronavirus

1. Introduction

The outbreak of COVID-19 has spread around the world and become a public health emergency of international concern. There have been nearly 60 million infections and over 1.4 million deaths reported as of November 24, 2020, since the first case, which was identified in December 2019. Though the majority of patients have mild disease (*1*), the large number of severe and critical cases pose great challenges to the global healthcare system.

Many studies have analysed the characteristics of severe COVID-19 and identified biomarkers for prognosis prediction (*2-4*). Drugs are urgently needed for both prophylaxis and the treatment of severely ill patients. A number of drugs that have been approved for other diseases are being tested for the treatment of COVID-19 patients, but there is an absence of data from appropriately designed clinical trials showing that these drugs, either alone or in combination, will prove effective (*5*). Particularly, some of the treatments (*i.e.*, drugs for malaria) are controversial and have caused heated discussion (*6*).

Therefore, as the percentage of severe cases has markedly decreased since March in Shanghai, China, multiple treatments, especially hydroxychloroquine, were retrospectively analysed to assess their efficacy in different disease stages of coronavirus disease 2019 (COVID-19).

2. Materials and Methods

2.1. Study design and participants

This retrospective cohort study included patients (≥ 15 years old) from Shanghai Public Health Clinical Center (Shanghai, China). All patients who were diagnosed with COVID-19 according to the Seventh Edition of the Guidance for COVID-19 of China (*7*) were screened, and those who died or were discharged between January 20, 2020 (*i.e.*, when the first patients were admitted), and April 30, 2020, were included in our study.

The study was approved by the Research Ethics Commission of Shanghai Public Health Clinical Center (V1.1-2020-02.08), and the requirement for informed consent was waived by the Ethics Commission.

2.2. Data collection

Epidemiological, demographic, clinical, laboratory, treatment, and outcome data were extracted from electronic medical records using a standardized data collection form, among which a modified version of the WHO/International Severe Acute Respiratory and Emerging Infection Consortium case record form was used for severe acute respiratory infections. All data were checked by two physicians (YYM and YS), and a third researcher (BJH) adjudicated any differences in interpretation between the two primary reviewers.

2.3. Laboratory procedures

Local Centers for Disease Control and Prevention was responsible for SARA-CoV-2 detection in respiratory specimens by real-time RT-PCR methods. Throat swab specimens and stool swab specimens were obtained for SARS-CoV-2 PCR re-examination every two or three days after the clinical remission of symptoms, including fever, cough, and dyspnoea, but only qualitative data were available. The criteria for discharge were absence of fever for at least 3 days, substantial improvement in chest CT, clinical remission of respiratory symptoms, and two throat swab samples and two stool swab samples negative for SARS-CoV-2 RNA obtained at least 24 h apart.

2.4. Treatment

All the patients were treated in strict accordance with the novel coronavirus infection diagnosis and treatment proposal formulated by the National Health Commission and Health Committee of China (*7*). Oral hydroxychloroquine was prescribed at 400 mg once a day for 10-14 days. Intravenous vitamin C was used at 5-15 g per day for at least 3 days. Subcutaneous thymosin alpha 1 (1.6 mg) was used three times per week. The dose of lopinavir-ritonavir was 0.5 g twice a day for 3-5 days, and arbidol was orally administered at 0.2 g three times a day for 5-7 days. Corticosteroid and immunoglobulin therapy were empirically administered as a combined regimen when severe pneumonia was diagnosed, and the common doses were 40 mg once or twice a day for 3 days and 10 g for 3-5 days, respectively. Low molecular weight heparin was prophylactically used when D-dimer gradually increased to prevent thrombotic events.

2.5. Definitions

The illness grade of COVID-19 was defined according to the Chinese management guideline for COVID-19 (version 7.0) (*7*). Mild disease includes patients with mild symptoms but no manifestation of pneumonia on imaging. Moderate disease includes patients with fever, cough, sputum production, and other respiratory tract or non-specific symptoms along with the manifestation of pneumonia on imaging. Severe pneumonia was defined as the presence of respiratory distress with respiratory frequency ≥ 30/min, SaO_2/SpO_2 below 94% on room air or a PaO_2 to FiO_2 ratio of 300 or lower. Critical disease includes respiratory failure and the need for mechanical ventilation, or shock or combination with other organ failure and the need treatment in an ICU. Disease aggravation indicates that (1) mild or moderate disease on admission progressed to severe or critical disease; or (2) severe disease on admission progressed to critical disease. Disease improvement indicates that the body temperature is lower than before, respiratory symptoms are relieved in mild patients, and lung CT or chest X ray show that the lesions appear to be more absorbed and dissipated than before in moderate, severe or critical patients. Treatments with the same drug were assigned to three groups: (1) not used indicates not starting the treatment; (2) not used early indicates not starting the treatment within 5 days of diagnosis or treatment not lasting for at least 3 days; and (3) used early indicates starting the treatment within 5 days of diagnosis and lasting for at least 3 days.

2.6. Statistical analysis

Depending on the distribution of the data, categorical variables are described as frequencies and percentages, and continuous variables are described as median and interquartile range (IQR) values. Binary logistic regression was used to screen the factors associated with disease aggravation in COVID-19 patients. The improvement time of hydroxychloroquine usage was estimated by the Kaplan-Meier method, and any differences in improvement time were evaluated with the log-rank test. Multivariable analyses with the Cox proportional hazards model were used to estimate the effects of prognostic factors on improvement time, days to negative throat swabs and days to negative stool

swabs. Statistical analyses were performed using SPSS 23.0 software (IBM, Armonk, NY, USA). The figures were constructed using GraphPad Prism 8.0.

3. Results

3.1. Clinical characteristics and outcomes of the study population

As of April 30, 2020, a total of 616 patients were enrolled in this study. The median age was 39 years (IQR, 28-56 years), and 342 (55.52%) patients were male (Table 1). A total of 172 patients (27.92%) had one or more coexisting chronic medical conditions. Hypertension (97 [15.75%]) was the most common comorbidity, followed by diabetes (40 [6.49%]). The Charlson Index was also calculated, and the Charlson Index of 99 patients (16.07%) was equal to or greater than 2. There were 138 mild, 470 moderate, 3 severe and 5 critical patients on admission, and the number increased by 50 for severe patients and 18 for critical patients on discharge. The percentage of severe and critical cases decreased from 22.36% before February 5 to 2.43% from March 6 to April 30 (Table 1). Up to the time of submission, a total of 608 (98.7%) patients were discharged, and 8 (1.3%) patients died.

3.2. Treatments of the study population

All of the patients were admitted to negative pressure isolation rooms. All patients were treated in strict accordance with the novel coronavirus infection diagnosis and treatment proposal formulated by the National Health Commission and Health Committee of China (*7*). As shown in Figure 1, hydroxychloroquine, vitamin C and thymosin alpha 1 were empirically used

Table 1. Clinical characteristics and outcomes

Disease grade on discharge	Mild *n* = 118 (%)	Moderate *n* = 430 (%)	Severe *n* = 50 (%)	Critical *n* = 18 (%)
Age				
≥ 65	0 (0.00)	45 (0.93)	20 (40.00)	6 (33.33)
< 65	118 (100.00)	385 (0.23)	30 (60.00)	12 (66.67)
Sex				
Male	68 (49.15)	228 (53.02)	31 (62.00)	15 (83.33)
Female	50 (42.37)	202 (46.98)	19 (38.00)	3 (16.67)
Comorbidity				
Hypertension	3 (2.54)	67 (15.58)	16 (32.00)	11 (61.11)
Diabetes	1 (0.85)	28 (6.51)	7 (14.00)	4 (22.22)
Cardiovascular disease	0 (0.00)	0 (0.00)	0 (0.00)	2 (11.11)
Chronic lung disease	0 (0.00)	15 (3.48)	1 (2.00)	2 (11.11)
Chronic kidney disease	0 (0.00)	4 (0.93)	0 (0.00)	2 (11.11)
Charlson Index				
< 2	115 (97.46)	343 (79.77)	34 (68.00)	5 (27.78)
≥ 2	3 (2.54)	87 (20.23)	16 (32.00)	13 (72.22)
Grade distribution by different time points				
Before Feb. 5 (*n* = 246)	4 (1.63)	187 (76.02)	41 (16.67)	14 (5.69)
Feb. 6 – Mar. 5 (*n* = 82)	4 (4.88)	72 (87.80)	4 (4.88)	2 (2.44)
Mar. 6 – Apr. 30 (*n* = 288)	110 (38.19)	171 (59.38)	5 (1.74)	2 (0.69)

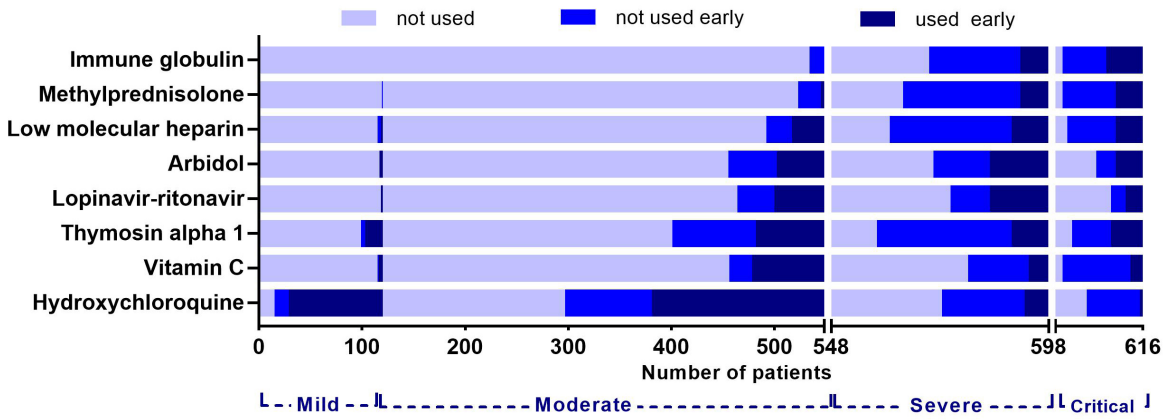

Figure 1. Multiple treatment for patients with different disease aggravation. Treatments that were used early in more than 62 (10%) patients includes hydroxylchloroquine, vitamin C, thymosin alpha 1, lopinavir-ritonavir and arbidol.

according to patient status decided by the attending physicians. Antiviral drugs (*e.g.*, lopinavir-ritonavir and arbidol) were administered to a small proportion of patients. Corticosteroid and methylprednisolone were not normally used unless they were considered necessary (*e.g.*, ARDS) in a panel discussion by experts.

3.3. Factors associated with disease aggravation

A binary logistic regression model was used to identify the factors associated with disease aggravation in patients with COVID-19. Treatments that were used early in 62 (10%) patients were included in this model. As shown in Figure 2, several independent variables were included in this model. Age ≥ 65 years old, thymosin alpha 1, lopinavir-ritonavir and arbidol used in early time were associated with the disease aggravation ($p < 0.05$) and early use of hydroxychloroquine was a protective factor associated with disease aggravation (95% CI: 0.040-0.575, $p = 0.006$).

3.4. Clinical improvement

A Cox proportional hazards model was used to identify the factors associated with improvement within 20 days in patients with COVID-19. As shown in Figure 3, the early use of hydroxychloroquine was the only factor that markedly reduced the improvement time ($p = 0.016$) compared with other treatments used early. Improvement time of different hydroxychloroquine usages were shown in Figure 4.

3.5. Viral clearance

In a Cox proportional hazards regression model, hydroxychloroquine used early was associated with earlier PCR conversion by 20 days in throat swabs (HR = 1.558, $p = 0.001$) and with earlier PCR conversion by 15 days in stool swabs (HR = 1.400, $p = 0.028$) (Figure 5).

4. Discussion

Currently, no specific therapeutic agents or preventive vaccines are available and approved for COVID-19. However, a number of drugs that have been approved for other diseases, some of which have been tried in patients with SARS-CoV and MERS-CoV, are being evaluated for the tresatment of COVID-19. These drugs include remdesivir, baricitinib, chloroquine, hydroxychloroquine, the interleukin-6 (IL-6) receptor monoclonal antibody tocilizumab, and the anti-influenza drugs favipiravir and umifenovir (*8,9*).

Hydroxychloroquine is capable of affecting several cellular pathways and therefore may have several mechanisms of action against SARS-CoV-2 (*10*). In addition, many findings suggest that hydroxychloroquine is effective at impairing SAR-CoV-2 replication *in vitro* (*11,12*). To date, several small studies and subjective reports have published evidence of the effectiveness of hydroxychloroquine for the prevention and treatment of COVID-19 (*13,14*). Furthermore, several official guidelines have already incorporated hydroxychloroquine as the suggested treatment of patients with COVID-19 (*15,16*). However, some articles were published for the ineffectiveness of hydroxychloroquine in the treatment of COVID-19 (*17,18*). Therefore, more clinical studies need to be performed to come to a definite conclusion.

Although adverse events were reported to be higher in hydroxychloroquine recipients than in non-recipients (*17*), another retrospective analysis of 1,061 cases in

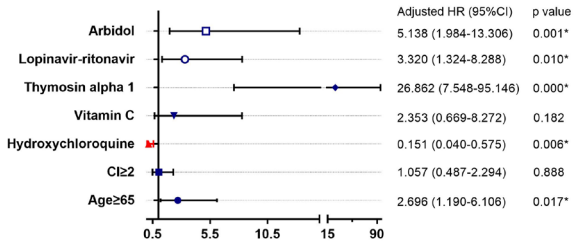

Figure 2. Effect of multiple treatments used early associated with disease aggravation.

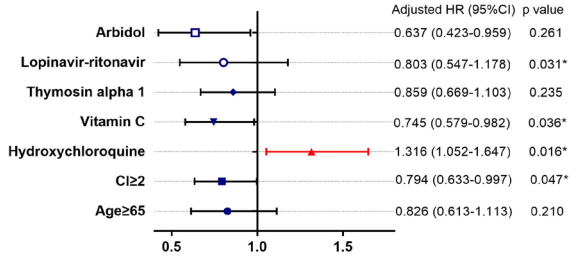

Figure 3. Effect of multiple treatments used early on the improvement time.

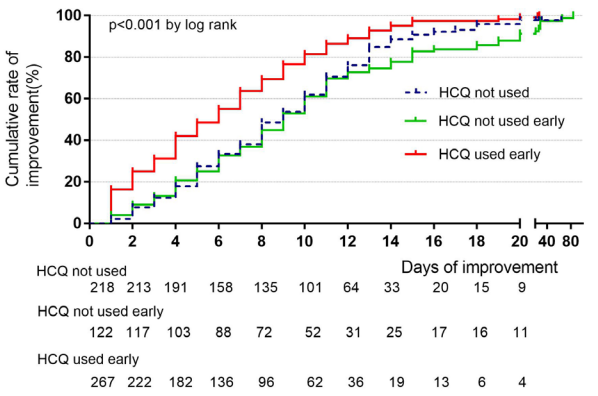

Figure 4. Improvement time of different hydroxychloroquine (HCQ) usages. Kaplan-Meier curves of the time to improvement in days with HCQ used early versus HCQ not used and HCQ not used early in the intention-to-treat population. Clinical improvement by 20 days was significantly different in patients with HCQ used early and with HCQ not used ($p = 0.016$, 95%CI: 1.052-1.647).The median time to clinical improvement was 6 days in the HCQ used early group, compared with 9 days in the without HCQ used group and 8 days in the with HCQ not used early group.

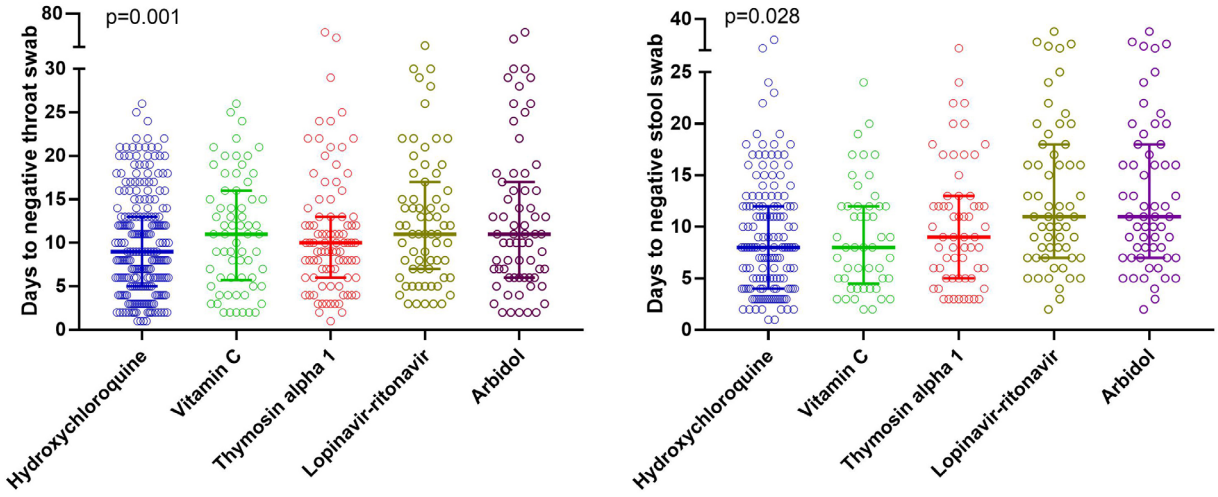

Figure 5. Days to negative results for throat swabs and stool swabs by different treatments used early.

France showed that a total of 2.3% of patients reported mild adverse events (gastrointestinal or skin symptoms, headache, insomnia and transient blurred vision) (*19*). Based on *in vitro* data, doses as high as 800 mg, if not higher, followed by 400 mg for several days, may be required for effective viral clearance in humans (*11*). Chinese official guidelines recommend 500 mg twice a day for 10 days and if severe gastrointestinal reactions occur, 500 mg once a day was administered (*16*). In our research, hydroxychloroquine was orally administered at 400 mg once a day for 10-14 days. As few adverse events were observed and no severe adverse events happened, hydroxychloroquine was recommended as safe with a low incidence of adverse events in patients.

Research has reported that the administration of hydroxychloroquine did not result in a significantly higher probability of negative conversion than the standard of care alone in patients with COVID-19 (*17*). However, in our research, the administration of hydroxychloroquine was specified. The early use of hydroxychloroquine, which is the use of hydroxychloroquine within 5 days of diagnosis, was found to decrease the swab PCR conversion days. Furthermore, the early use of hydroxychloroquine was also found to prevent the progression of the disease aggravation from mild and moderate to severe and critical. The use of hydroxychloroquine at an early stage is a potentially life-saving therapeutic strategy both to treat and cure patients before irreversible severe respiratory complications occur. Though hydroxychloroquine was found to prevent disease aggravation in our study, it was not significantly associated with a decrease in in-hospital mortality (*18*). Therefore, in the late stage of COVID-19 disease, hydroxychloroquine was not recommended as a life-saving treatment.

Though in our study, the early use of thymosin alpha 1 and high-dose intravenous VC was not associated with preventing disease progression and shortened improvement time because of the limitation of grouping and statistics, they were not considered ineffective for COVID-19. Thymosin alpha 1 has been used experimentally, as it has been used in the treatment of viral infections as an immune response modifier for many years, and thymosin alpha 1 supplement has been reported to significantly reduce the mortality of severe COVID-19 patients (*20*). Hemila and colleagues reported that various high-dose intravenous VC infusions (*e.g.*, 200 mg/kg body weight/day, divided into 4 doses) shortened the intensive care unit (ICU) stay by 7.8%, accompanied by a significant reduction in the mortality rate (*21*). Various high-dose intravenous VC infusions (doses varying between 5 g and 15 g per day) have been successfully used in the treatment of moderate to severe COVID-19 patients in China. Given that high-dose VC is safe, well-designed clinical studies are needed for severe and critical cases. Articles suggested that therapies (*i.e.*, ritonavir plus lopinavir) directed at viral replication may prove to be more effective in the early stages of COVID-19 before significant pneumonia symptoms have developed (*5*). However, this conclusion was not drawn in our research. On the one hand, there were few patients who underwent this treatment in our study; on the other hand, the combination of ritonavir plus lopinavir was reported to not provide sufficient benefits over standard care, including the reduction of viral RNA load (*22*). Therefore, future trials in patients with severe illness may help to confirm or exclude the possibility of a treatment benefit.

In conclusion, the use of hydroxychloroquine at an early stage is a potential therapeutic strategy for treating patients before irreversible severe respiratory complications occur. The early use of hydroxychloroquine decreased the improvement time and the duration of COVID-19 detection in throat and stool swabs.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Bijie Hu, Department of Infectious Diseases, Department of Hospital Infection Management Zhongshan Hospital of Fudan

University, 180 Fenglin Road, Shanghai 200032, China.
E-mail: hu.bijie@zs-hospital.sh.cn

Hongzhou Lu, Department of Infectious Diseases, Shanghai Public Health Clinical Center, 2901 Caolang Road, Shanghai 201508, China.
E-mail: luhongzhou@fudan.edu.cn

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Original Article

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Comparison of the surgical outcomes in patients with synchronous versus metachronous multiple hepatocellular carcinoma

Yutaka Midorikawa[1,*], Tadatoshi Takayama[1], Tokio Higaki[1], Osamu Aramaki[1], Kenichi Teramoto[1], Nao Yoshida[1], Yusuke Mitsuka[1], Shingo Tsuji[2]

[1] Department of Digestive Surgery, Nihon University School of Medicine, Tokyo, Japan;
[2] Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan.

SUMMARY Multiplicity is one of the characteristics of hepatocellular carcinoma (HCC), and patients with multiple HCC (≤ 3 nodules) are recommended as candidates for liver resection. To confirm the validity of resecting multiple HCC, we compared the surgical outcomes in patients with synchronous and metachronous multiple HCC. Patients who underwent resection for multiple HCC (2 or 3 nodules) were classified into the "synchronous multiple HCC" group, while those undergoing resection for solitary HCC and repeated resection for 1 or 2 recurrent nodules within 2 years after initial operation were classified into the "metachronous multiple HCC" group. After one-to-one matching, longer operation time and more bleeding were seen in the synchronous multiple HCC group (n = 98) than those in the metachronous multiple HCC group (n = 98); however, the complication rates were not different between the two groups. The median overall survival times were 4.0 years (95% CI, 3.0-5.9) and 5.9 years (4.0-NA) for the synchronous and metachronous multiple HCC (after second operation) groups, respectively (P = 0.041). The recurrence-free survival times were shorter in the synchronous multiple HCC group than in the metachronous multiple HCC group (median, 1.5 years [95% CI, 0.9-1.8] versus 1.8 years, [1.3-2.2]) (P = 0.039). On multivariate analysis, independent factors for overall survivals in the synchronous multiple HCC group were older age, cirrhosis, larger tumor, and tumor thrombus. Taken together, resection of metachronous multiple HCC still has good therapeutic effect, even better than synchronous multiple HCC, so resection is suggested for metachronous multiple HCC.

Keywords multiple hepatocellular carcinoma, patient stratification, guideline

1. Introduction

Multiplicity is one of the characteristics of hepatocellular carcinoma (HCC) (*1*), and patients with multiple HCC, classified as the intermediate stage (B) in the Barcelona-Clinic Liver Cancer staging classification, are candidates for transcatheter arterial chemoembolization (TACE) (*2*). In contrast, the survival benefit of liver resection for multiple HCC has been reported (*3-5*); survival of patients undergoing liver resection for such nodules was longer than that of patients undergoing TACE according to a nationwide study (*6*) and a prospective study (*7*). Consequently, resection of multiple HCC ≤ 3 is indicated by clinical practice guidelines for hepatocellular carcinoma in Japan (*8*).

However, the surgical outcomes of patients with multiple HCC after resection were worse than those of patients with solitary nodule even if they met the criteria regarding the number of tumors and liver function (*9,10*). Therefore, patients with multiple HCC should not be treated in the same way, but should be stratified for determination of the candidates of liver resection.

Multiple HCC consist of primary HCC and its metastatic nodules or new lesions (*11-13*); therefore, multiple HCC can be considered as having "synchronous multiple HCC". On the other hand, patients with recurrent HCC could be considered as having other nodules after resection of primary HCC; therefore, such patients can be considered as having "metachronous multiple HCC" (*14*). Besides of synchronous multiple HCC, therefore, solitary HCC harbors the potential of multiplicity.

In this study, we compared the surgical outcomes of patients with synchronous multiple HCC to those of patients with metachronous multiple HCC after propensity matching and identified the types of multiple HCC patients that were good candidates for liver resection.

2. Patients and Methods

2.1. Patients

Patients who underwent initial and curative resection of HCC between 2000 and 2018 at the Nihon University Itabashi Hospital (Tokyo, Japan) were included in this study. Each participant provided written informed consent, and this study was approved by the review board of Nihon University. All clinical investigations were conducted according to the principles of the Declaration of Helsinki.

2.2. Multiple HCC

Patients who underwent liver resection for multiple HCC (2 or 3 nodules) were defined as "synchronous multiple HCC". In contrast, patients who underwent liver resection for solitary HCC and repeated resection for 1 or 2 recurrent nodules were defined as "metachronous multiple HCC". Considering the malignant potential of multiple nodules, patients who showed recurrence 2 years after the initial operation were excluded from the metachronous HCC group. Survival after the first operation for the synchronous multiple HCC group and that after the second operation for the metachronous multiple HCC group were compared after propensity-score matching to adjust for patient background, liver function, and tumor status, including age, sex, hepatitis viral infection, alcohol abuse, diabetes mellitus, varices, Child-Pugh classification, indocyanine green clearance rate at 15 min (ICGR15), background liver, and status of the main tumor. Considering that the tumor number was different during the operation between the two groups, tumor markers were not included as covariates. Propensity scores were matched using a caliper width of 0.2, and one-to-one pair matching was performed.

2.3. Indications for liver resection

The indications for liver resection and other treatments for patients with HCC were determined by assessing their liver functional reserve according to the Guidelines on Liver Cancer Examination and Treatment in Japan (*8*). Briefly, patients with Child-Pugh A or B with up to 3 lesions were candidates for liver resection.

2.4. Surgical procedure

Open liver resection was performed in all patients according to the criteria based on the liver function (*15*). The liver was transected under ultrasonographic guidance using the clamp-crushing method with the inflow-blood-occlusion technique (*16*). Curative resection was defined as the complete removal of recognizable viable HCC diagnosed preoperatively or intraoperatively with macroscopically tumor-free surgical margins. Postoperative complications were stratified according to the Clavien-Dindo classification (*17*), which defines morbidities as complications with a score of ≥ 3a. Complications specific to liver resection were defined as described previously (*18*).

2.5. Follow-up after operation

All patients were followed up to determine the postoperative recurrence as described previously (*19*). Briefly, levels of tumor markers, including alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP), were measured and imaging studies, including computed tomography and ultrasonography, were performed every 3 months in all patients. Recurrence was diagnosed by dynamic computed tomography and/or magnetic resonance imaging. The date of recurrence was defined as the date of examination when the recurrent HCC was noted.

2.6. Statistical analysis

Data collected from each group were statistically analyzed using the Fisher's exact test and Wilcoxon rank-sum test. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. Prognostic factors for overall survival were identified using the Cox proportional hazards regression model. P value < 0.10 was set as the cut-off value for elimination. The following 16 variables, considered potential confounders, were examined: age (≥ *vs.* < 75 years), sex, positive result for hepatitis B and C viruses, alcohol abuse, diabetes mellitus, Child-Pugh classification (5 *vs.* 6 or 7), ICGR15 (≥ *vs.* < 15%), esophageal varices, serum AFP level (≥ *vs.* < 100 ng/mL), serum DCP level (≥ *vs.* < 100 mAU/mL), and pathological findings of the main tumor (maximal tumor size [≥ *vs.* < 3.0 cm], vascular invasion of tumor, liver cirrhosis, tumor differentiation grade [poorly vs well and moderately], and surgical margin). P values < 0.05 were considered to indicate significance.

3. Results

3.1. Patients

A total of 1,244 patients underwent initial and curative resection of HCC. After excluding 39 patients with four or more nodules, 1,205 patients were classified as those with multiple nodules (2 or 3 nodules) (the synchronous multiple HCC group, n = 280) and those with solitary HCC (n = 925). After excluding 493 patients who showed no recurrence within 2 years and 323 patients who did not undergo liver resection for recurrent HCC, the remaining 109 patients were classified as the metachronous multiple HCC group (Figure 1). The median of disease-free interval from the initial

operation in the metachronous multiple HCC group was 1.3 years (range, 0.2-1.9 years).

Before propensity-score matching, more patients were males (P = 0.020) and had higher serum DCP level (P = 0.025), larger main tumor (P = 0.001), and more frequent liver cirrhosis (P = 0.017) in the synchronous multiple HCC group (Table 1).

3.2. Operative data

After one-to-one matching, the operation time was longer (P = 0.039) with more bleeding (P = 0.015) in the synchronous multiple HCC group (n = 98) than that in the metachronous multiple HCC group (n = 98) owing to the number of resected tumors (Table 2). The postoperative stay was longer in the synchronous multiple HCC group (P < 0.001). The frequencies of overall complications and morbidities were not significantly different between the two groups. One patient in the synchronous multiple HCC group underwent re-operation for intraperitoneal abscess, and three patients in the metachronous multiple HCC group for intraperitoneal hemorrhage (two patients) and bile leakage (one patient). There was no hospital death in this series.

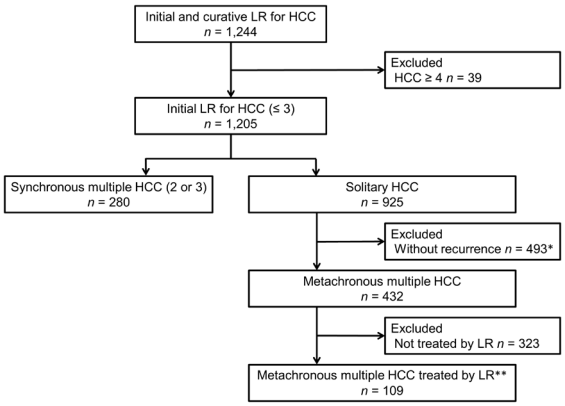

Figure 1. Flow diagram showing patient recruitment and follow-up. *, Including 88 patients with recurrence, 2 years after initial liver resection. **, Primary and recurrent HCC were treated by liver resection in these patients. LR, liver resection; HCC, hepatocellular carcinoma.

3.3. Survivals

After a median follow-up of 3.2 years (range, 0.2-12.8 years), a total of 150 patients (76.5%) had recurrence, and treatment for recurrent HCC did not differ between the two groups (Table 3). The median overall survival times were 4.0 years (95% confidence interval [CI], 3.0-5.9) and 5.9 years (4.0-NA) for the synchronous multiple HCC (n = 98) and metachronous multiple HCC (n = 98) (after second operation) groups, respectively (P = 0.041) (Figure 2A). The recurrence-free survival was shorter in the synchronous multiple HCC group (median, 1.5 years; 95% CI, 0.9-1.8) than that in the metachronous multiple HCC group (1.8 years, 1.3-2.4; P = 0.039) (Figure 2B). The 5-year-rates of overall survivals were 43.2% and 60.0% in the two groups, respectively, and those of recurrence-free survivals were 10.7% and 15.6%, respectively.

On multivariate analysis, the independent factors for overall survivals in the synchronous multiple HCC group (n = 280) were older age (hazard ration [HR], 1.57; 95% CI, 1.13-2.19, P = 0.006), liver cirrhosis (HR, 1.67; 1.12-2.48, P = 0.010), larger tumor (HR, 1.84; 95% CI, 1.30-

Table 1. Patient background

	Before propensity-score matching			After propensity-score matching		
	Synchronous (n = 280)	Metachronous (n = 109)	P	Synchronous (n = 98)	Metachronous (n = 98)	P
Age, years	69 (33-85)	68 (40-82)	0.341	67 (33-78)	68 (40-82)	0.597
Sex, male	236 (84.2)	80 (73.3)	0.020	76 (77.5)	77 (78.5)	1
Hepatitis B	43 (15.3)	20 (18.3)	0.540	16 (16.3)	17 (17.3)	1
Hepatitis C	152 (54.2)	56 (51.3)	0.651	56 (57.1)	52 (53.0)	0.666
Alcoholic	85 (30.3)	28 (25.6)	0.362	24 (24.4)	28 (28.5)	0.627
Diabetes mellitus	92 (32.8)	29 (26.6)	0.272	27 (27.5)	26 (26.5)	1
Child-Pugh, 5	214 (76.4)	83 (76.1)	1	75 (76.5)	74 (75.5)	1
ICGR15, %	13.2 (3.1-59.1)	12.6 (1.9-44.9)	0.726	12.8 (4.3-59.1)	12.8 (1.9-44.9)	0.691
Varices	64 (22.8)	24 (22.0)	0.893	21 (21.4)	21 (21.4)	1
Alpha fetoprotein, ng/mL	24 (1-211,062)	18 (1-541,432)	0.147	20 (1-211,602)	14 (1-541,432)	0.178
DCP, mAU/mL	108 (8-75,000)	44 (8-75,000)	0.025	71 (8-75,000)	44 (8-75,000)	0.287
Pathology†						
Tumor size, cm	3.5 (0.9-21.0)	2.7 (0.9-16.5)	0.001	3.1 (1.2-19.0)	2.9 (1.0-16.5)	0.297
Differentiation, por	42 (15.0)	10 (9.1)	0.139	12 (12.2)	11 (11.2)	1
Vascular invasion	89 (31.7)	43 (23.6)	0.058	30 (30.6)	26 (26.5)	0.635
Surgical margin, positive	20 (7.1)	9 (8.2)	0.673	9 (9.1)	8 (8.1)	1
Cirrhosis	116 (41.4)	31 (37.7)	0.017	32 (32.6)	31 (31.6)	1

Data are presented as median (range) or n (%). †, Histological findings of the main tumor. ICGR15, indocyanine green clearance rate at 15 minutes; DCP, desgamma-carboxy prothrombin.

Table 2. Operative data

	Synchronous (n = 98)	Metachronous (n = 98)	P
Operative time, min	346 (184-691)	328 (146-631)	0.039
Bleeding, mL	345 (25-2,988)	257 (15-1,900)	0.015
Transfusion	6 (6.1)	5 (5.1)	1
Complications			
Overall	40 (40.8)	30 (30.6)	0.179
Morbidity	30 (30.6)	22 (22.4)	0.257
Liver failure	1 (1.0)	0	
Intraperitoneal hemorrhage	0	2 (2.0)	
Bile leakage	2 (2.0)	3 (2.0)	
Intraperitoneal abscess	1 (1.0)	0	
Ascites	0	1 (1.0)	
Infection (Wound, Drainage tube)	14 (14.2)	5 (5.1)	
Respiratory	8 (8.1)	11 (11.2)	
Ileus	1 (1.0)	0	
Others	3 (3.0)	0	
Re-operation	1 (2.5)	3 (3.2)	0.621
Operative death	0	0	1
Hospital stay, days	14 (7-99)	12 (7-36)	< 0.001

Data are presented as median (range) or n (%). Data at the second operation for the patients with metachronous multiple HCC.

Table 3. Treatment for recurrent HCC

	Synchronous (n = 77)	Metachronous (n = 73)	P
Liver resection	25 (32.4)	30 (41.0)	0.311
Radiofrequency ablation	3 (3.8)	2 (2.7)	1
TACE/HAIC	43 (55.8)	36 (49.3)	0.513
Radiation	1 (1.2)	2 (2.7)	0.612
Chemotherapy	1 (1.2)	2 (2.7)	0.612
None	3 (3.8)	1 (1.3)	0.620

Data are presented as n (%). Data at the second operation for the patients with metachronous multiple HCC. TACE, transcatheter arterial chemoembolization; HAIC, hepatic arterial infusion chemotherapy

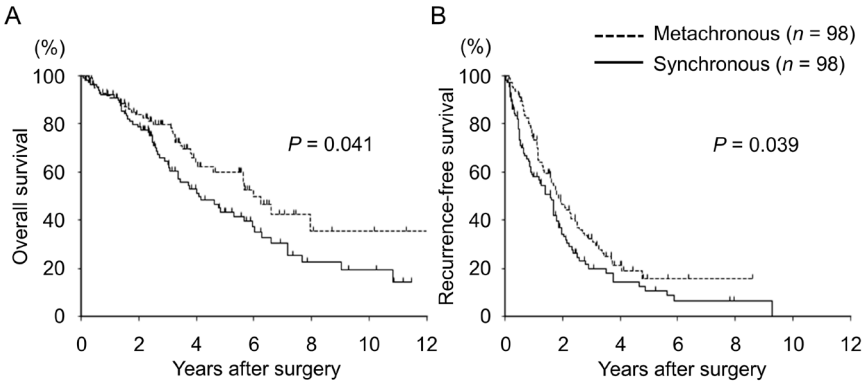

Figure 2. Survival of patients with multiple HCC. (A) Overall survival of patients in the synchronous multiple HCC group is significantly shorter than that of patients in the metachronous multiple HCC group (P = 0.041). **(B)** Recurrence-free survival of patients in the synchronous multiple HCC group is significantly shorter than that of patients in the metachronous multiple HCC group (P = 0.039).

2.63, P < 0.001), and tumor thrombus (HR, 1.42; 1.01-2.07, P = 0.041) (Table 4). The median overall survival times were significantly shorter in patients ≥ 70 years old (3.4 years [range, 3.0-4.1 years] versus 6.2 years [4.0-7.1 years], P = 0.022), those with cirrhosis (3.4 years [range, 3.0-4.1 years] versus 6.2 years [4.0-7.1 years], P = 0.006), and those with tumor ≥ 3.0 cm (3.4 years [range, 3.0-4.3 years] versus 5.6 years [4.1-6.6 years], P = 0.006), and shorter in patients with tumor thrombus (2.8 years [range, 2.1-4.0 years] versus 4.7 years [3.9-5.7 years], P = 0.074) (Figure 3).

4. Discussion

Our data showed that the survival of patients with synchronous multiple HCC after liver resection was

Table 4. Prognostic factors for survival of patients with synchronous multiple HCC

	Univariate			Multivariate		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Age	1.44	1.05-1.99	0.023	1.57	1.13-2.19	0.006
Sex	0.91	0.61-1.41	0.684			
Hepatitis B	0.67	0.39-1.07	0.100			
Hepatitis C	0.91	0.66-1.25	0.575			
Alcohol	0.98	0.69-1.37	0.913			
Diabetes mellitus	0.86	0.61-1.21	0.408			
Child-Pugh	1.38	0.96-1.94	0.073	1.19	0.81-1.73	0.351
ICGR15	1.43	1.03-1.98	0.028	1.22	0.85-1.75	0.263
Varices	1.45	1.02-2.04	0.037	1.21	0.79-1.82	0.362
Alpha fetoprotein	0.98	0.67-1.39	0.924			
DCP	0.90	0.65-1.23	0.518			
Cirrhosis	1.55	1.12-2.13	0.006	1.67	1.12-2.48	0.010
Tumor size	1.57	1.13-2.19	0.006	1.84	1.30-2.63	< 0.001
Thrombus	1.35	0.96-1.88	0.080	1.42	1.01-2.07	0.041
Differentiation grade	1.14	0.81-1.61	0.444			
Surgical margin	0.80	0.35-1.55	0.549			

CI, confidence interval; ICGR15, indocyanine green clearance rate at 15 minutes; DCP, des-gamma-carboxy prothrombin.

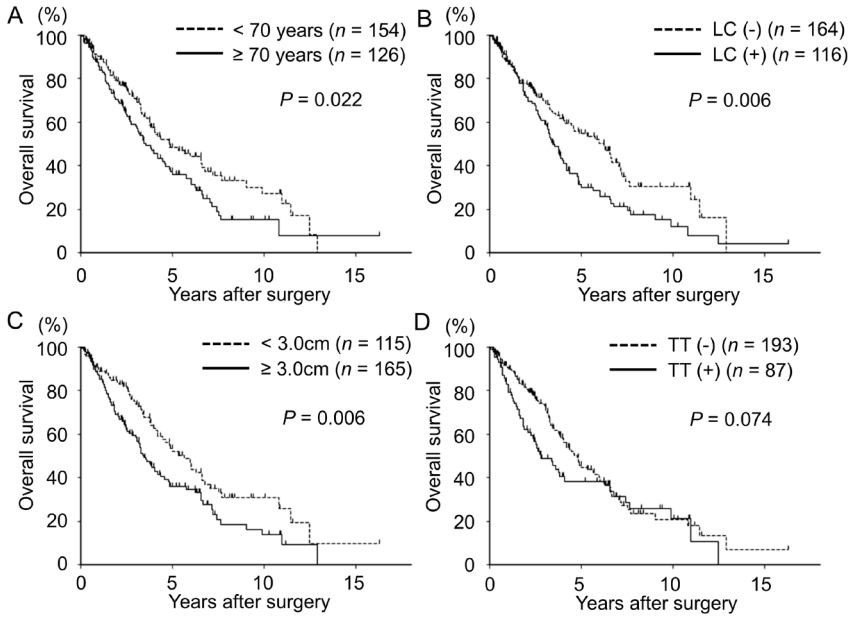

Figure 3. Overall survival of patients in the synchronous multiple HCC group. (A) Survival in patients ≥ 70 years of age versus that in patients < 70 years of age (P = 0.022). **(B)** Survival in patients with liver cirrhosis versus that in patients without cirrhosis (P = 0.006). **(C)** Survival in patients with the tumor ≥ 3.0 cm versus that in patients with the tumor < 3.0 cm (P = 0.006). **(D)** Survival in patients with tumor thrombus versus that in patients without tumor thrombus (P = 0.074). LC, liver cirrhosis; TT, tumor thrombus.

shorter than that of patients with metachronous multiple HCC. Therefore, patients with metachronous multiple HCC (≤ 3 nodules) are good candidates for resection (*20*).

We and others previously reported that surgical outcomes of patients with multiple HCC were worse than those of patients with solitary HCC (*9,10*). Many patients with solitary HCC were alive without recurrence for long periods after operation (*21-23*). In this study, we defined the tumors that recurred within 2 years after operation of solitary HCC as metachronous multiple HCC because such tumors harbor the potential of multiple nodules. We compared the surgical outcomes of patients with multiple HCC and those with solitary HCC with potential of multiplicity.

The survival times of the metachronous multiple HCC group was defined as the period from the date of second operation to that of recurrence or death, as usually applied for comparison of survivals between the synchronous and metachronous liver metastasis of colorectal cancer (*24,25*). This is because one of the tumors in the synchronous multiple HCC is an intrahepatic metastasis from the primary HCC or *de*

novo HCC, which develops after appearance of the primary HCC. Consequently, the surgical outcomes of patients with synchronous multiple HCC were worse than those even after recurrence in patients with solitary HCC.

The occurrence of multiple HCC can be explained by intrahepatic metastasis or multicentric origin (*11-13*). To adjust the two mechanisms of hepatocarcinogenesis between the synchronous and metachronous multiple HCC groups, only patients with the disease-free interval < 2 years were included in the latter group in this study. This could be because intrahepatic metastasis is observed within two years after initial resection in many patients with metachronous multiple HCC by genome analysis using a next-generation sequencer (*14,26*). Consequently, the solitary HCC patients who were cured by liver resection were excluded (*27*), and we assumed that the inclusion criteria regarding disease-free interval was appropriate.

The patient background differed between the two groups. As the metachronous multiple HCC group had candidates who underwent repeated resection of recurrent HCC, liver cirrhosis was less frequent before propensity matching. Consequently, the complication rates were higher in the synchronous multiple HCC group. Therefore, the complication rates were not different after matching of the background. The status of the main tumor was more advanced in the synchronous multiple HCC group, and tumor conditions may be matched between the two groups. On the other hand, tumor number was different between the two groups at the initial operation, which must affect the serum tumor marker levels; therefore, they were not matched in this series. Further, the synchronous multiple HCC group showed longer operation time and more bleeding even after propensity matching owing to the difference in the number of resected tumors.

On multivariate analysis, the survival of patients with synchronous multiple HCC was shorter in the older patients with larger tumors, tumor thrombus, and liver cirrhosis. Given that surgical outcomes of patients with multiple HCC are not preferable (*9,10*), the candidates for liver resection should be determined based on the patient background, tumor status, and liver function (*20*); however, studies have showed the superiority of liver resection to TACE for multiple HCC (*6,7*).

This study had several limitations. First, there is no consensus for definition of metachronous multiple HCC. For example, solitary HCC followed by recurrence within six months or one year might have been considered synchronous multiple HCC as in the metastasis of colorectal cancer. On the other hand, metachronous multiple HCC in this study was a counterpart of the synchronous multiple HCC; therefore, we defined the two types of multiple HCC in the Methods section. Next, this study was a retrospective study, and selection bias, especially in the determination of candidates for surgery for recurrent HCC, might have affected the surgical outcomes in the metachronous multiple HCC group. Finally, multiple HCC are divided into the two categories; intrahepatic metastasis and multicentric origin. However it is clinically difficult to distinguish between the two types of multiple HCC, and the frequencies of intrahepatic metastasis and multicentric origin in each group are unknown, which might affect the surgical outcomes in this study.

In conclusion, the surgical outcomes of patients with synchronous multiple HCC, usually same as "multiple HCC", were worse even after curative resection. By contrast, resection of metachronous multiple HCC still had good therapeutic effect, so resection is suggested for metachronous multiple HCC.

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**Address correspondence to:*
Yutaka Midorikawa, Department of Digestive Surgery, Nihon University School of Medicine, 30-1, Oyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan.
E-mail: mido-tky@umin.ac.jp

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Original Article

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Subcuticular sutures reduce surgical site infection after repeat liver resection: a matched cohort analysis

Shintaro Yamazaki, Tadatoshi Takayama*, Yoritaka Matsuno, Yusuke Mitsuka, Nao Yoshida, Masamichi Moriguchi, Tokio Higaki

Department of Digestive Surgery, Nihon University School of Medicine, 30-1 Ohyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan

SUMMARY Liver cancer frequently requires repeated liver resections due to the high recurrence rate. The aim of this study was to clarify whether subcuticular sutures reduce wound complication rates following repeat incisions. Data from 382 repeated liver resections in 1,245 consecutive patients were assessed. Patients were divided into a Subcuticular sutures group and a Skin staples group on the basis of the wound-closure method. To avoid bias in analysing wound complications, data were matched to adjust for patient background and operation variables. After matching, 82 matched, paired patients with subcuticular sutures or skin staples were compared. Total wound complication rate was significantly lower with subcuticular sutures than with skin staples (8.5% *vs.* 20.7%, p = 0.027). Incisional surgical site infection was also lower with subcuticular sutures than with skin staples (6.1% *vs.* 17.1, p = 0.028). Univariate analysis revealed 4 factors associated with wound complications: body mass index; serum albumin concentration; wound length; and closure with skin staples. Multivariate analysis revealed closure with skin staples (odds ratio, 2.91; 95% confidence interval, 1.07-7.94; p = 0.037) as the only independent factor negatively associated with wound complications. Subcuticular sutures appear to reduce wound complications compared to skin staples following repeat incision for liver resection.

Keywords surgical site infection, subcuticular suture, repeat liver resection

1. Introduction

Using subcuticular sutures in wound closure reportedly contributes to reducing wound complication rates (*1,2*). However, subcuticular sutures have shown superiority only in sub-group analyses of clean-contaminated surgeries, such as digestive surgeries (*3,4*).

In hepatobiliary surgery, the wound complication rate is still over 10% and is considered to contribute to prolonged hospitalization (*5-7*). Furthermore, liver cancers such as hepatocellular carcinoma and colorectal metastasis show a high frequency of recurrence and around 30-50% of patients require repeat laparotomy (*8,9*). Re-laparotomy using the same incision is thus frequent, and has been considered to be negatively associated with wound complications. Attempts to minimize wound complications have included intraoperative administration of antimicrobials, irrigation of wounds before closure, use of absorbable sutures in the subcutaneous layer and application of wound-protective dressing materials. However, none of these steps have proven particularly effective (*10-12*).

No studies have focused on factors associated with wound complications following repeated incisions for liver resection. This may be because the development of wound complications is a multifactorial issue, involving factors such as operation scale, procedures, background of chronic liver diseases, longer operation times and greater length of the surgical incision for thoracotomy. We therefore analysed factors associated with wound complications after adjusting for perioperative variables to minimize selection bias.

2. Materials and Methods

2.1. Study design

Between April 2001 and December 2015, data were collected from 1,245 consecutive patients who underwent hepatic resection for liver cancer. The target for analysis was repeated laparotomy using the same incision in patients undergoing skin closure with either subcuticular sutures or metallic skin staples, without use of subcutaneous drainage tubes. Subcutaneous drainage tubes were avoided because we have previously applied this method to minimize wound complications (*7*).

Also, the patients who have large incisional hernia caused by previous operation was excluded from this study. Patients were divided into groups according to the use of subcuticular sutures or skin staples for wound closure.

2.2. Wound closure

A J-shaped incision was the most common incision, with thoracotomy added only when the tumor was located in the posterior segment or caudate lobe. At wound closure, surgical gloves and instruments were changed and the subcutaneous space was irrigated with saline. Routine approximation of the fat layer with 3-0 multifilament absorbable suture (polyglycolic acid, OPEPOLYX® N; Alfresa Pharma Corporation, Tokyo, Japan) was performed before skin closure. Finally, 3-0 monofilament absorbable sutures (polydioxanone, MONODIOX®; Alfresa Pharma Corporation) were used for subcuticular sutures with both interval and length of bite of sutures set at 15 mm. In the skin staples group, metallic skin staples (Appose ULC 35W; Medtronic, Tokyo, Japan) 10 mm in size were used. Peripheral blood circulation in the scar tissue was poor and may thus create poor conditions for tissue adaption. To remove the tissue of poor local skin blood circulation, the scar tissue was routinely removed at incision in case of repeat laparotomy.

2.3. Assessment

The total incidence of all wound complications was compared between the Subcuticular and Skin staples groups before and after matching. Finally, factors associated with wound complications were analysed and whether subcuticular sutures were superior to skin staples in repeated incision was assessed.

2.4. Definitions

According to the Centers for Disease Control and Prevention guideline (*13*), superficial incisional surgical site infection (SSI) was defined as an infection occurring within 30 days of surgery and arising only in the skin or subcutaneous tissue. Deep incisional SSI was defined as infection occurring within 30 days of surgery and arising only in the fascial or muscle layers. Wound complications were defined as the presence of signs relating to treatment: wound disruption, stitch abscess, abscess, seroma or hematoma, or superficial or deep incisional SSI. All information on wound complications was collected on a uniform sheet in our unit.

2.5. Data manipulation for matching

First, raw preoperative data for patient characteristics, tumor status and operation-related variables were compared between groups to assess wound complications. Next, biases in background data were examined, then data were matched using Greedy matching methods (*14*) to the data of nearest-neighbor patients. Written informed consent for clinical analysis was obtained from each patient undergoing liver resection. All analyses were performed in accordance with the ethics guidelines for clinical studies at Nihon University Itabashi Hospital (RK-170214-03).

2.6. Statistical analyses

Data are expressed as medians and ranges or as absolute values and percentages. Student's *t*-test, the $\chi 2$ test, and Fisher's exact test were used for univariate analyses, as appropriate. Continuous variables were compared using the Mann-Whitney *U*-test. Multivariate analysis was performed using logistic regression methods. Odds ratios with 95% confidence intervals derived from logistic regression analysis were calculated. Values of $P < 0.05$ were considered to indicate statistical significance. All analyses were performed using JMP version 13.2 statistical software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Study flow

Between April 2001 and December 2015, a total of 1,245 patients underwent hepatic resection for liver cancer at our institution (Figure 1). Among these, 863 patients were excluded from analysis because of primary operation for liver resection. Next, 116 patients were excluded because of wound closure methods that were neither subcuticular sutures nor metallic skin staples. Then, the remaining 266 patients were divided into two groups according to the method of wound closure. To eliminate the influence of background, matching was performed before analysis. Finally, 82 patients from each group were assessed and comparisons were made between subcuticular sutures (Subcuticular sutures group) and metallic skin staples (Skin staples group).

3.2. Raw data analysis and matching

Before matching, neither patient background characteristics nor liver functional reserve differed significantly between groups (Table 1). However, the rate of hepatitis C infection was significantly higher in the Skin staples group (31.5%) than in the Subcuticular sutures group (14.8%, $p = 0.004$). As for operation-related variables, median wound length was significantly longer in the Subcuticular sutures group (36 cm, range, 20-56 cm) than in the Skin staples group (31 cm, 22-48 cm; $p = 0.039$).

Operation-related variables such as wound length differed markedly between groups, and so were matched

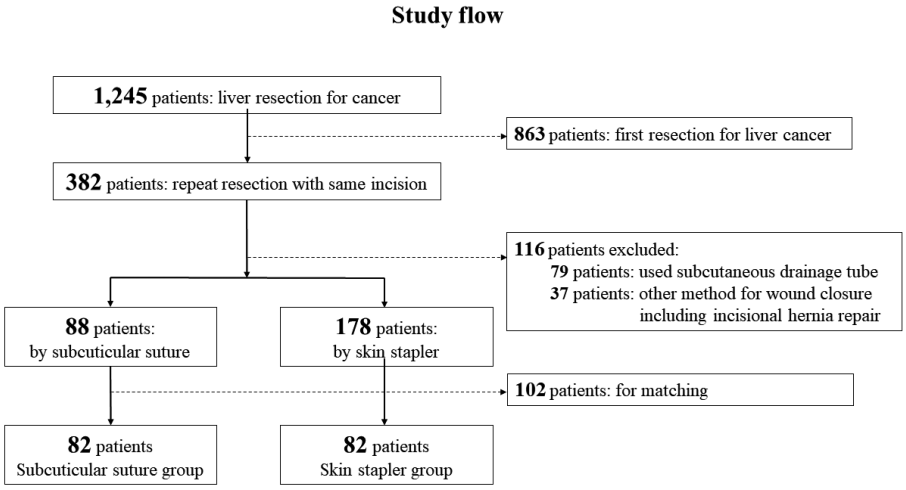

Figure 1. Flow diagram of the patients. A total of 1,245 patients underwent hepatic resection for liver cancer at our institution. 863 patients were excluded from analysis because of primary operation for liver resection. Finally, 82 patients from each group were assessed and comparisons were made between subcuticular sutures (Subcuticular sutures group) and metallic skin staples (Skin staples group).

Table 1. Raw patient's baseline characteristics

	Subcuticular stures (n = 88)	Staples (n = 178)	p-value
Age (year)	68 (44-79)	69 (26-82)	0.671
Body Mass Index	22.7 (15.8-32.3)	23.1 (15.6-30.8)	0.611
Diabetes mellitus	27 (30.7)	60 (33.7)	0.621
Anticoagulation therapy	13 (14.8)	34 (19.1)	0.384
Preoperative chemotherapy	23 (26.1)	32 (18.0)	0.122
History of smorking	13 (14.8)	33 (18.5)	0.445
ICG-R15 (%)	10.7 (1.9-33.3)	11.7 (2.5-32.7)	0.411
Viral infection			
HBV	20 (22.7)	30 (16.9)	0.249
HCV	13 (14.8)	56 (31.5)	0.004
Total-bilirubin (mg/dL)	0.73 (0.20-1.59)	0.59 (0.25-1.77)	0.748
Prothrombin time (%)	100 (80-100)	100 (74-100)	0.299
Albumin (g/dL)	4.3 (2.5-5.2)	4.2 (3.1-5.7)	0.584
Child-pugh class A	84 (95.5)	174 (97.8)	0.302
Bile leakage	8 (9.8)	8 (9.8)	1.000
HbA1c (%)	5.9 (4.6-8.8)	5.8 (4.1-8.8)	0.831
Platelet count (10^4μL)	17.4 (7.8-45.8)	17.8 (6.3-44.1)	0.671
Creatinine (mg/dL)	0.88 (0.36-1.49)	0.76 (0.48-1.44)	0.142
Diseases			
Hepatocellular carcinoma	47 (53.4)	105 (59.0)	0.387
Operation related variables			
Segmentectomy	9 (10.2)	20 (11.2)	0.804
Lobectomy or extented lobectomy	8 (9.1)	13 (7.3)	1.000
Operation time (min)	341 (165-681)	361 (126-812)	0.698
Blood loss (mL)	249 (2-1114)	261 (10-945)	0.682
Wound length (cm)	36 (20-56)	31 (22-48)	0.039
Wound thickness (mm)	21 (6-37)	22 (6-47)	0.516
Bile leakage	8 (9.8)	18 (10.1)	0.792
With thoracotomy	37 (42.1)	84 (47.2)	0.427
Clavien-Dindo classification grade (≥ IIIb)	3 (3.4%)	5 (2.8%)	0.746

ICG-R15, indocyanine green retention rate at 15 minutes; HBV, Hepatitis B virus; HCV, Hepatitis C virus.

for to avoid bias (Table 2). After matching, no significant differences in background status and operation-related variables of patients were identified.

3.3. Wound complications

In matched groups, the total complication rate was significantly lower in the Subcuticular sutures group (8.5%) than in the Skin staples group (20.7%, p = 0.027) (Table 3). Incisional SSI was significantly less frequent in the Subcuticular sutures group (6.1%) than in the Skin staples group (17.1%, p = 0.028). Rates of other wound complications did not differ significantly between groups.

Table 2. Baseline characteristics after matching

	Subcuticular stures (n = 82)	Staples (n = 82)	p-value
Age (year)	68 (44-79)	69 (26-80)	0.683
Body Mass Index	22.6 (16.2-32.3)	22.3 (15.6-30.8)	0.545
Diabetes mellitus	27 (32.9)	27 (32.9)	1.000
Anticoagulation therapy	12 (14.6)	13 (15.9)	0.828
Preoperative chemotherapy	20 (24.4)	14 (17.1)	0.248
History of smorking	13 (14.8)	16 (19.5)	0.539
ICG-R15 (%)	10.8 (1.9-32.8)	11.6 (2.5-32.5)	0.240
Viral infection			
HBV	18 (22.0)	9 (11.0)	0.058
HCV	12 (14.6)	19 (23.2)	0.163
Total-bilirubin (mg/dL)	0.60 (0.20-1.69)	0.60 (0.25-1.65)	0.772
Prothrombin time (%)	100 (80-100)	100 (74-100)	0.219
Albumin (g/dL)	4.3 (2.5-5.1)	4.2 (3-5.7)	0.594
Child-pugh class A	80 (97.6)	78 (95.1)	0.406
HbA1c (%)	5.9 (4.6-8.8)	5.9 (4.8-8.0)	0.934
Platelet count (10^4μL)	17.5 (7.8-45.8)	18.6 (6.3-39.1)	0.668
Creatinine (mg/dL)	0.81 (0.37-1.45)	0.79 (0.48-1.44)	0.066
Diseases			
Hepatocellular carcinoma	39 (47.6)	43 (52.4)	0.520
Operation related variables			
Segmentectomy	9 (11.0)	12 (14.6)	0.483
Lobectomy or extented lobectomy	5 (6.1)	5 (6.1)	1.000
Operation time (min)	339 (165-656)	329 (134-805)	0.785
Blood loss (mL)	242 (2-1114)	238 (10-945)	0.631
Wound length (cm)	35 (20-51)	34 (23-47)	0.488
Wound thickness (mm)	21 (6-37)	21 (7-46)	0.693
Bile leakage	8 (9.8)	8 (9.8)	1.000
With thoracotomy	36 (43.9)	38 (46.3)	0.754
Clavien-Dindo classification grade (≥ IIIb)	3 (3.7%)	3 (3.7%)	1.000

ICG-R15, indocyanine green retention rate at 15 minutes; HBV, Hepatitis B virus; HCV, Hepatitis C virus.

Table 3. Complications

	Subcuticular sutures (n = 82)	Staples (n = 82)	Odds ratio (95%CI)	p-value
Total wound complication rate	7 (8.5%)	17 (20.7%)	2.80 (1.09-7.18)	0.027
Incisional SSI	5 (6.1%)	14 (17.1%)	3.17 (1.08-9.26)	0.028
Organ space SSI	1 (1.2%)	2 (2.4%)	2.03 (0.18-22.78)	0.560
Wound separation	0 (0%)	0 (0%)	0	1.000
Heaematoma	1 (1.2%)	1 (1.2%)	1.00 (0.06-16.26)	1.000
Seroma	1 (1.2%)	2 (2.4%)	2.03 (0.18-22.78)	0.560

SSI, surgical site infection.

3.4. Risk analysis

In univariate analysis, body mass index (BMI) (p = 0.033), serum albumin value (p = 0.031), wound length (p = 0.023) and closure with skin staples (p = 0.027) were the factors predicting wound complications (Table 4). In multivariate analysis, the only factor showing a negative independent association with wound complications was closure with skin staples (odds ratio, 2.91; 95% confidence interval, [1.07-7.94], p = 0.037).

4. Discussion

Significant differences in complication rates were observed with subcuticular sutures compared to skin staples, especially for incisional SSI. This study thus suggested the superiority of subcuticular sutures as a standard procedure in repeated incisions for liver resection. To the best of our knowledge, this study is the first to target repeated incisions, which has been considered a key risk factor for wound complications. 24 We have therefore conducted a study focusing on repeated liver resection as a potential risk factor for the development of wound complications.

Wound complication rates remain high, within the range of 6-33%, and have yet to show any substantial reductions in open liver surgery over the last decade (*5-7,10,15*). Even though wound complications were not severe and did not affect patient survival, such complications cause discomfort in patients, prolong

Table 4. Logistic regression analysis for the risk of wound complication

Risk factor	Univariate analysis			Multivariate analysis		
	odds ratio	95%CI	*p*-value	odds ratio	95%CI	*p*-value
Patient's related variables						
Age (≥ 75 years-old)	1.34	0.45-3.95	0.596	1.94	0.57-6.63	0.290
Gender (male)	2.51	0.71-8.92	0.142			
Body Mass Index (≥ 25)	2.62	1.06-6.48	0.033			
Wound closure (Staples)	2.80	1.09-7.18	0.027	2.91	1.07-7.94	0.037
Albumin (< 3.5 g/dL)	4.86	1.01-23.25	0.031	4.36	0.74-25.58	0.102
Child-pugh class A	3.09	0.53-17.90	0.187			
Creatinine (≥ 1.00 mg/dL)	0.97	0.26-3.58	0.961			
Presence of diabetes mellitus	1.27	0.52-3.11	0.606			
ICG-R15 (≥ 15%)	2.41	0.98-5.94	0.051			
Preoperative chemotherapy	1.01	0.35-2.93	0.989			
Operation related variables						
Lobectomy or extented lobectomy	1.50	0.30-7.53	0.620	0.97	0.16-5.66	0.969
Operation time (≥ 300 min)	2.46	0.87-6.97	0.083	2.44	0.64-9.34	0.193
Blood loss (≥ 300 mL)	2.13	0.89-5.10	0.086	1.67	0.64-4.38	0.299
Subcutaneous drainage	1.15	0.44-2.98	0.780			
With thoracotomy	1.87	0.78-4.49	0.159			
Wound length (≥ 35 cm)	2.88	1.13-7.39	0.023	2.08	0.67-6.43	0.202
Wound thickness (≥ 30 mm)	1.55	0.47-5.11	0.469	0.92	0.24-3.47	0.902
Bille leakage	0.82	0.17-3.85	0.799			

SSI, surgical site infection.

hospitalization and increase the total medical cost (*7*). Regarding SSI in the abdominal space, we have successfully reduced abdominal space infection based on results from two clinical studies (*16,17*). In contrast, a randomized controlled trial we performed to examine the effects of a subcutaneous drainage tube (UMIN000004437) on wound complications failed to reduce wound complications, and identified no specific factors related to wound complications (*7*). Because the development of wound complications is multifactorial, operation-related factors need to be considered along with systemic diseases such as diabetes merits, chronic hepatitis and metabolic disease. Moreover, nutritional status may affect wound adaptation in cases of liver cirrhosis. Using the matched cohort method, factors associated with wound complications were adjusted in the present study before analysis.

In multivariate analysis, use of skin staples was the only independent factor negatively associated with wound complications. In the present study, the total wound complication rate in the Subcuticular group was 8.5%, appearing preferable to the 13.1% (34/260 patients) in our previous study using a subcutaneous drainage tube (*7*). We speculated that peripheral blood circulation was poor for the scar tissue and may thus have created suboptimal conditions for wound adaption in repeated resection. In such situations, layer-to-layer fixation such as subcuticular sutures may help reduce wound complications (*2-4*).

In univariate analysis, BMI and wound length were predictors of wound complications. These results support minimally invasive hepatobiliary surgeries such as laparoscopic liver resection as one possible option to reduce wound complication rates. Indeed, the rate of severe complications was similar between open and laparoscopic surgeries, while the total wound complication rate was lower and operation time was shorter with laparoscopic surgery than with open surgery (*18-20*). Laparoscopic surgery is thus one feasible option when the tumour extension is preferable for laparoscopic surgery.

In our previous study, we focused on reducing the subcutaneous accumulation of effusions (*7*). However, we found no preventive effects of subcutaneous drain placement against wound infection. Because the effusion of subcutaneous tissue was smaller than we expected. Thus, we routinely performed subcutaneous suturing of the fat layer using absorbable sutures before skin closure for all patients to reduce dead space in subcutaneous tissue. Especially, the peripheral blood circulation in the repaired connective tissue may be interrupted by repeat incision and may thus create poor conditions for tissue adaption. Using with subcuticular sutures, the tightened tissue space may have contributed to reduced bacterial penetration into the subcutaneous tissue, especially with thick and long incisions.

Limitations of this study were that: *i*) randomization was not performed because of the retrospective nature of the study; and *ii*) laparoscopic surgeries were not included. Therefore, we are now initiating the largest RCT regarding wound closure in liver resection, to confirm the superiority of subcutaneous suture (UMIN000036670).

In conclusion, subcuticular sutures can be recommended as a standard procedure for wound closure following repeated incision in open liver surgery.

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**Address correspondence to:*
Tadatoshi Takayama, Department of Digestive Surgery, Nihon University School of Medicine, 30-1 Ohyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan.
E-mail: takayama.tadatoshi@nihon-u.ac.jp

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The C-reactive protein to albumin ratio is an excellent prognostic predictor for gallbladder cancer

Yongjin Bao, Junsheng Yang, Yunfei Duan, Yuxiang Chen, Weibo Chen*, Donglin Sun*

Department of Hepatopancreatobiliary Surgery, The Third Affiliated Hospital of Soochow University, Changzhou, China.

SUMMARY A number of inflammation indicators based on C-reactive protein (CRP) and albumin have been widely used to predict the prognosis in several types of tumors, but their functions in gallbladder cancer (GBC) have rarely been explored. The aim of our study is to evaluate and compare the prognostic values of the C-reactive protein to albumin ratio (CAR), Glasgow prognostic score (GPS), modified Glasgow prognostic score (mGPS) and high-sensitivity modified Glasgow prognostic score (HS-mGPS) in patients with GBC. 144 GBC patients who received curative surgery in our hospital from January 2010 to May 2017 were enrolled in this research. The Kaplan-Meier analysis showed that the median OS of the patients in the high CAR group was significantly shorter than the patients in the low group ($p < 0.001$), and higher scores of GPS, mGPS and HS-mGPS were also associated with decreased OS, respectively. However, according to the Receiver Operating Characteristic (ROC) curve, the CAR was superior to the other prognostic scores in determining the prognosis for the GBC patients. In the multivariate analysis, CAR was verified as an independent risk factor for poor prognosis, together with tumor differentiation, T stage and postoperative complications. All in all, compared to the other three CRP-albumin-related prognostic predictors, CRA is a better indicator in predicting poor long-term outcomes in GBC patients after radical surgery.

Keywords gallbladder cancer, C-reactive protein to albumin ratio, prognostic score, surgery

1. Introduction

Gallbladder cancer (GBC) is one of the most frequently diagnosed malignancies of the biliary system and ranks as the sixth most common cancer of the digestive system, notorious for its poor prognosis (*1-3*). According to the latest global cancer statistical analysis in 2018, about 219,000 cases were diagnosed with GBC, while 165,000 people died of it worldwide (*4*). Because of its nature of insensitivity to radiotherapy and chemotherapy, surgical resection is still the only possible curative treatment for GBC (*2,5*). However, due to the lack of effective early diagnostic methods and typical symptoms, most patients are diagnosed at advanced stages. Even with aggressive surgical intervention and other comprehensive therapies, the 5-year survival rate is still below 20% (*6*). Currently, risk factors associated with the prognosis of GBC include tumor stage, pathological grade, lymph node metastasis, vascular and nerve invasion, and tumor margin status (*7-9*). However, the above indicators can only be obtained after surgery. Therefore, it is important to find a simple and reliable preoperative risk factor to predict the prognosis of GBC.

Recently, more and more research has shown that the occurrence of tumors is closely related to inflammatory response. Inflammatory cytokines in the tumor microenvironment, such as interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (*10*), amplify the inflammatory effect and promote tumor growth by recruiting inflammatory cells to the tumor location. Therefore, many predictors based on inflammation indicators are widely adopted to predict the prognosis of various tumors (*11-13*). Among those immune-nutritional parameters, some C-reactive protein-albumin-related indexes have been identified as important prognostic factors in cancer patients, such as the C-reactive protein to albumin ratio (CAR), Glasgow prognostic score (GPS), modified Glasgow prognostic score (mGPS) and high-sensitivity modified Glasgow prognostic score (HS-mGPS) (*11,14-16*). Although the prognostic significance of those inflammation-based markers have been confirmed in a variety of tumors, their role in GBC is rarely reported. Therefore, this retrospective cohort study aimed to determine the prognostic effects of those inflammatory indicators and compare their predictive values in GBC patients after radical surgery.

2. Materials and Methods

2.1. Patients

We retrospectively reviewed 144 cases of resectable GBC patients registered between January 2010 and May 2017 who received curative surgery in the Third Affiliated Hospital of Soochow University. We collected all the patients' clinical characteristics, including demographics, degree of tumor differentiation, lymph node metastasis, pathologic TNM staging, laboratory data and follow-up data. The hematological parameters including C-reactive protein, albumin, Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 were extracted from blood samples within 1 week prior to surgery (the upper physiological values of CEA and CA19-9 are 10 U/mL and 37 U/mL) (*17*). TNM staging of GBC was described according to the 8th edition of the American Joint Committee on Cancer (AJCC-8th) manual. The inclusion criteria were as follows: *i*) patients with histological confirmed GBC; *ii*) patients who received radical surgery; and *iii*) patients aged > 18 years old. The exclusion criteria were: *i*) patients without complete clinical data or follow-up data; *ii*) patients with other malignancies; *iii*) patients with perioperative or non-neoplastic death; and *iv*) patients with acute inflammation, infectious diseases or autoimmune diseases.

An informed consent was obtained from all patients and the study was approved by the Ethics Committee of The Third Affiliated Hospital of Soochow University.

2.2. Definition of the inflammation-based prognostic scores

In this research, the CAR was calculated as the serum CRP level divided by the serum albumin level. According to the ROC curve and the Youden index, the optimal cut-off value of CAR was 0.069. The value of GPS was calculated as follows: patients with both an elevated CRP level (> 10 mg/L) and hypoalbuminemia (< 35 g/L) were allocated a score of 2; patients with only one of the above-mentioned abnormalities were allocated a score of 1, and those without these abnormalities were allocated a score of 0. In addition, the mGPS score was defined as follows: patients with both high CRP level (> 10 mg/L) and low albumin (< 35 g/L) got a score of 2; patients with only a high CRP level got a score of 1 and those without a high CRP value regardless of albumin level got a score of 0. When it came to HS-mGPS, the threshold of CRP was set as 3 mg/L according to the report by Proctor (*18*).

2.3. Surgical strategy and follow-up

For patients at T1a stage, simple cholecystectomy could reach radical resection. For patients at T1b and T2 stage, we routinely perform a cholecystectomy and liver wedge-resection with a margin of 2 cm around the gallbladder and lymph node dissection at station N1. For patients at T3 stage, the gallbladder combined with liver S4b + S5 segment resection and lymph node dissection at station N2 were performed. For some patients at T4 stage, on the basis of radical resection of GBC, combined resection of affected organs and enlarged regional lymph node dissection were performed. In cases of incidental GBC, patients with stage T1b-T4 received reoperation and radical resection. Follow-up was performed by the outpatient clinics or phone calls, and the end of the follow-up period was the last follow-up visit (October 2020) or death.

2.4. Statistical analysis

The receiver operating characteristic (ROC) curve was used to determine the optimal cut-off values of the CAR and evaluate the performance of each prognostic score, and the areas under the curve (AUC) were measured and compared using the method established by DeLong *et al.* (*19*) χ^2 test , Mann-Whitney *U* test and Kruskal-Wallis test were used to determine the statistical associations of the clinicopathological factors. The primary outcome measure of this study was overall survival (OS), from the date of surgery to the date of death or last follow-up. The survival curve was constructed using the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate analyses were performed to evaluate the impact of the variables to OS in all patients. Variables shown to have significant prognostic value by univariate analysis were further assessed by multivariate analysis using a Cox's proportional hazards model. A *p*-value < 0.05 was considered to be a significant difference in all analyses. All statistical analyses were performed by SPSS version 20.0.

3. Results

3.1. Patient characteristics

The demographic characteristics of the 144 patients with GBC are shown in Table 1. Among these patients, 97 patients were female and 37 were male, the median age was 63 years. According to the pathological findings, the resected specimens included 82 (56.9%) well- or moderately- and 62 (43.1%) poorly-differentiated tumors. 27 (18.7%) of tumors had nerve and 42 (29.2%) had liver invasion. Referring to the AJCC-8th TNM staging of GBC, 24 (16.7%), 60 (41.7%), 51 (35.4%) and 9 (6.2%) tumors staged T1, T2, T3 and T4, respectively, and 100 (69.4%), 37 (25.7%) and 7 (4.9%) tumors staged N0, N1 and N2 stage, respectively. Finally, 55 (38.2%) tumors were classified as stages I-II and 89 (61.8%), III-IV. In regard to surgical complications, a total of 38 (26.4%) patients had postoperative complications, including bile

Table 1. Correlations between the clinicopathologic parameters and each prognostic predictor

Factor	CAR		*p* value	GPS			*p* value	mGPS			*p* value	HS-mGPS			*p* value
	< 0.069	≥ 0.069		0	1	2		0	1	2		0	1	2	
Age			0.709[a]				0.155[b]				0.273[b]				0.341[a]
≤ 60	11	24		25	6	4		29	2	4		13	16	6	
> 60	38	71		58	34	17		80	11	18		42	37	30	
Gender			0.869[a]				0.071[b]				0.843[b]				0.942[a]
Male	13	24		16	17	4		27	6	4		15	13	9	
Female	36	71		67	23	17		82	7	18		40	40	27	
Differentiation			0.456[a]				0.355[a]				0.254[b]				0.081[b]
Well/moderate	30	52		50	23	9		65	8	9		30	36	16	
Poor	19	43		33	17	12		44	5	13		25	17	20	
T stage			0.020[c]				0.047[c]				0.294[c]				0.095[c]
T1	14	10		18	5	1		21	2	1		14	8	2	
T2	21	39		35	13	12		44	3	13		21	21	18	
T3	11	40		28	16	7		39	5	7		17	21	13	
T4	3	6		2	6	1		5	3	1		3	3	3	
N stage			0.146[c]				0.771[c]				0.147[c]				0.048[c]
N0	39	61		58	30	12		79	9	12		42	37	21	
N1	9	28		22	7	8		26	2	9		12	15	10	
N2	1	6		3	3	1		4	2	1		1	1	5	
TNM stage			< 0.001[a]				0.054[b]				0.011[b]				0.001[a]
I+II	30	25		38	13	4		48	3	4		31	16	8	
III+IV	19	70		45	27	17		61	10	18		24	37	28	
BMI (kg/m^2)			0.447[a]				0.232[b]				0.493[b]				0.169[a]
< 25	37	66		59	26	18		77	7	19		42	33	28	
≥ 25	12	29		24	14	3		32	6	3		13	20	8	
Nerve invasion			0.151[a]				0.221[b]				0.559[b]				0.305[a]
Present	6	21		12	11	4		19	4	4		7	11	9	
Absent	43	74		71	29	17		90	9	18		48	42	27	
Liver invasion			0.005[a]				0.291[b]				0.972[b]				0.079[a]
Present	7	35		23	15	4		31	7	4		11	21	10	
Absent	42	60		60	25	17		78	6	18		44	32	26	
Complications			0.018[a]				< 0.001[a]				0.070[b]				0.002[a]
Present	7	31		8	20	10		24	4	10		14	7	17	
Absent	42	64		75	20	11		85	9	12		41	46	19	
Serum CEA level (ng/mL)			0.097[a]				0.713[a]				0.222[b]				0.312[a]
< 5	39	63		61	27	14		81	7	14		43	35	24	
≥ 5	10	32		22	13	7		28	6	8		12	18	12	
Serun CA 19-9 level (U/mL)			0.688[a]				0.385[a]				0.197[b]				0.910[a]
< 37	27	49		42	20	14		53	8	15		28	28	20	
≥ 37	22	46		41	20	7		56	5	7		27	25	16	

BMI: body mass index; CAR: C-reactive protein to albumin ratio; CA 19-9: carbohydrate antigen 19-9; CEA: carcinoembryonic antigen; GPS: Glasgow prognostic score; HS-mGPS: high-sensitivity modified Glasgow prognostic score; mGPS: modified Glasgow prognostic score. [a]: Chi-square test; [b]: Mann-Whitney *U* test; [c]: Kruskal-Wallis test.

leakage in 11 (7.6%), abdominal abscess in 7 (4.9%), incision infection in 4 (2.8%), postoperative bleeding in 3 (2.1%), and lung infection in 14 (9.0%).

3.2. Prognostic role of the CRP-albumin-related indicators

As shown in Table 1, 49 patients were classified as the low CAR group (CAR < 0.069) and 95 patients as the high CAR group (CAR ≥ 0.069). Patients in the high CAR group tended to have postoperative complications ($p = 0.018$), liver invasion ($p = 0.005$) and advanced tumor status such as T stage ($p = 0.020$) and TNM stage ($p < 0.001$). Of the 144 patients, 83 had a GPS of 0, 40 had a GPS of 1 and 21 had a GPS of 2. The GPS was significantly correlated with T stage ($p = 0.047$) and postoperative complications ($p < 0.001$). In subgroup analysis of the mGPS, the 109 patients had a mGPS score of 0, 13 had a score of 1, and another 22 had a score of 2. There was significant association between higher score of mGPS and advanced TNM stage ($p = 0.011$). In contrast, the numbers of patients in HS-mGPS 0/1/2 was 55, 53 and 36, respectively. A higher score of HS-mGPS was associated with higher N stage ($p = 0.048$), TNM stage ($p = 0.001$) and postoperative complications ($p = 0.002$).

3.3. Survival analysis

During the follow-up, 101 patients died and 43 patients were censored at the last follow-up. According to the Kaplan-Meier analysis, the median OS of the patients

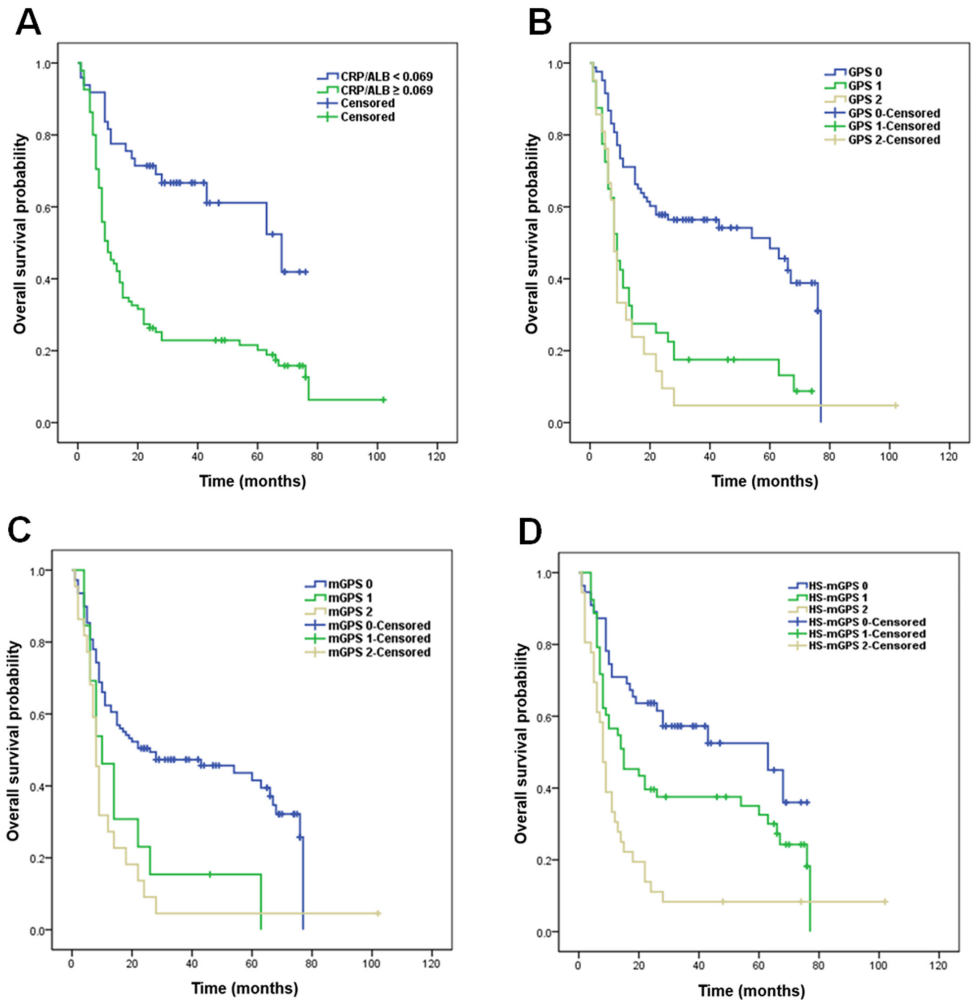

Figure 1. Kaplan–Meier curve demonstrated significant prognostic difference among the 144 GBC patients who underwent curative resection according to the (A) CAR, (B) GPS, (C) mGPS and (D) HS-mGPS.

in the high CAR group was significantly shorter than the patients in the low CAR group ($p < 0.001$, Figure 1A). Meanwhile, higher scores of GPS, mGPS and HS-mGPS were also correlated with worse prognosis ($p < 0.001$; Figure 1B, 1C, and 1D). Univariate analysis demonstrated that nerve invasion, tumor differentiation, liver invasion, T stage, N stage, and TNM stage were significantly correlated with shorter OS. Moreover, CAR, GPS, mGPS, HS-mGPS, postoperative complications, serum CEA level and CA 19-9 level were all associated with reduced OS in univariate analysis. The multivariate analysis showed that tumor differentiation, T stage, CAR and postoperative complications were independent prognostic factors of GBC (Table 2).

3.4. Comparison of prognostic performance among each prognostic score

The ROC curve demonstrated that the CAR was superior to the other prognostic scores in predicting 1- and 3-year OS. The AUC values of CAR at 1 year (0.785) and 3 years (0.798) was significantly higher than GPS (1 year: 0.684, $p = 0.0122$; 3 years: 0.615, $p < 0.0001$), mGPS (1 year: 0.644, $p < 0.0001$; 3 years: 0.601, $p < 0.0001$), and HS-mGPS (1 year: 0.684, $p = 0.0019$; 3 years: 0.704, $p = 0.0080$) (Figure 2A and 2B).

4. Discussion

Currently, the 8th edition of the TNM staging system for tumors released by AJCC is considered to be the most practical prognostic indicator of GBC. Nevertheless, some experts have suggested that the staging system lacks individual specificity because it focuses too much on the anatomical extent of disease and ignores the effects of biological factors (*20,21*). In this study, we revealed that CAR is an effective predictor of OS and superior to the other CRP-albumin-related indicators in predicting the prognosis of the GBC patients after radical surgery.

Actually, the development of tumors is a complex process, not only dependent on the biological features of tumor cells, but also closely related to the host's inflammatory response. It has already been well recognized that inflammation stimulates tumor progression and metastasis (*22,23*). In the case of GBC,

Table 2. Univariate and multivariate analysis for overall survival

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Age (years)		0.309		
≤ 60	1.000			
> 60	1.272 (0.800-2.021)			
Gender		0.079		
Female	1.000			
Male	0.673 (0.433-1.046)			
BMI (kg/m^2)		0.212		
< 25	1.000			
≥ 25	1.312 (0.857-2.011)			
Nerve invasion		< 0.001		0.747
Absent	1.000		1.000	
Present	2.471 (1.560-3.916)		0.902 (0.482-1.687)	
Differentiations		< 0.001		0.022
Well/moderate	1.000		1.000	
Poor	2.226 (1.419-3.322)		1.754 (1.083-2.840)	
Liver invasion		< 0.001		0.421
Absent	1.000		1.000	
Present	2.388 (1.401-3.666)		1.085 (0.467-2.413)	
T stage		< 0.001		< 0.001
T1+T2	1.000		1.000	
T3+T4	2.845 (2.190-3.695)		4.374 (2.323-8.236)	
N stage		< 0.001		0.240
N0	1.000		1.000	
N1	2.681 (1.711-4.198)		1.482 (0.845-2.601)	
N2	4.170 (1.866-9.317)		1.961 (0.727-5.289)	
TNM stage		< 0.001		NG
I+II	1.000			
III+IV	3.543 (2.675-7.413)			
CAR		< 0.001		0.003
< 0.069	1.000		1.000	
≥ 0.069	2.706 (1.474-5.148)		2.647 (1.434-5.560)	
GPS		< 0.001		0.070
0	1.000		1.000	
1	2.658 (1.697-4.162)		0.347 (0.134-0.902)	
2	3.125 (1.820-5.364)		1.435 (0.151-13.602)	
mGPS		< 0.001		0.143
0	1.000		1.000	
1	1.715 (0.984-3.631)		0.915 (0.335-2.500)	
2	2.528 (1.529-4.179)		2.039 (0.784-5.302)	
HS-mGPS		< 0.001		0.108
0	1.000		1.000	
1	1.772 (1.077-2.917)		1.217 (0.494-2.385)	
2	3.360 (1.993-5.666)		2.253 (1.076-5.541)	
Complications		< 0.001		0.028
Absent	1.000		1.000	
Present	3.688 (2.401-5.653)		1.741 (0.543-4.723)	
Serum CEA level		0.012		0.273
< 5	1.000		1.000	
≥ 5	1.735 (1.147-2.626)		0.742 (0.435-1.265)	
Serum CA 19-9 level		0.009		0.756
< 37	1.000		1.000	
≥ 37	1.805 (1.142-2.644)		1.085 (0.650-1.809)	

BMI: body mass index; CAR: C-reactive protein to albumin ratio; CA 19-9: carbohydrate antigen 19-9; CEA: carcinoembryonic antigen; CI: Confidence interval; GPS: Glasgow prognostic score; HR: Hazard ratio; HS-mGPS: high-sensitivity modified Glasgow prognostic score; mGPS: modified Glasgow prognostic score.

chronic cholecystitis always causes DNA damage and repeated tissue proliferation releases cytokines and growth factors, which induce canceration of cells. Therefore, the increase of peripheral blood inflammation indicators could reflect the inflammatory response status caused by tumor growth and invasion to a certain extent.

CRP is an acute phase response protein produced by the liver and regulated by a variety of pro-inflammatory cytokines. It is an important response indicator of the body's non-specific inflammatory response and an increased CRP is associated with

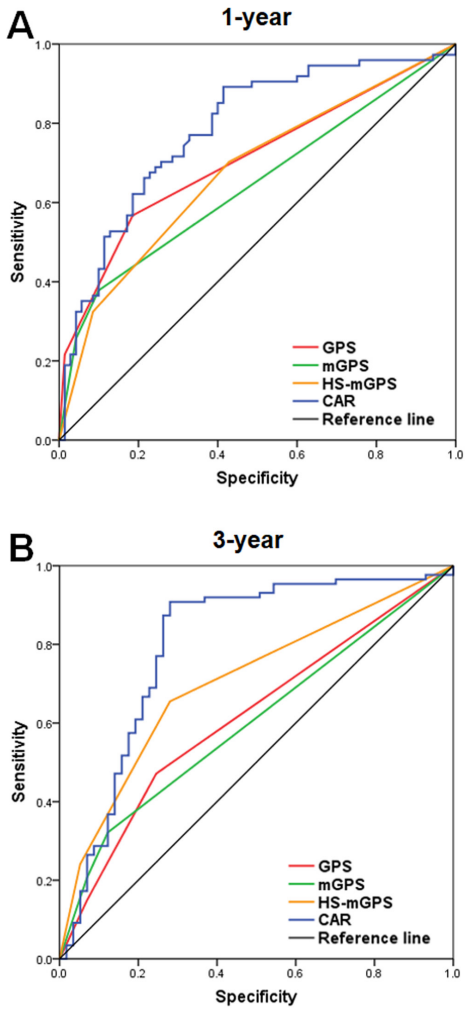

Figure 2. Comparison of the areas under the ROC curves for outcome prediction among the four CRP-albumin-related prognostic scores (CAR, GPS, mGPS, and HS-mGPS) at (A) 1 year and (B) 3 years.

unsatisfactory prognosis in a variety of tumors (*24,25*). In addition to CRP, serum albumin is also involved in systemic inflammation and hypoproteinemia is always considered to be a sign of poor long-term survival in cancer patients (*26,27*). The GPS is an objective indicator reflecting the inflammatory response and nutritional status, which combines the serum CRP value and albumin levels. It was first used to predict the prognosis of patients with lung cancer (*28*). Later, Inoue *et al.* (*29*) modified the prognostic score and named mGPS. Proctor *et al.* (*30*) found that the mGPS is superior to other inflammation-based prognostic scores such as NLR, PLR and PNI in predicting the prognosis of patients with various tumors. Furthermore, in a retrospective study by Tomoyuki *et al.* (*15*), they proved that a high preoperative mGPS was an independent prognostic indicator of poor survival in GBC. However, in this study, the mGPS has limited value in predicting the prognosis of patients with GBC. We found that in addition to the mGPS, the other three indicators are all related to the occurrence of postoperative complications. We believe the major reason is that only a small number of patients have CRP values greater than 10 mg/L, thus, it may be difficult to clarify the relationship between the mGPS and each variable. In order to solve the uneven distribution of preoperative parameters, Proctor *et al.* proposed HS-mGPS, which has a lower CRP threshold (> 3 mg/L). There is some research reported that the HS-mGPS is a better prognostic indicator than mGPS (*16,31,32*), and this conclusion is also verified in our study.

Recently, much research demonstrated that the CAR is significantly correlated with tumor progression and plays a vital function in assessing the prognosis of tumor patients (*14,33*). Although the CAR is also composed of CRP and albumin, the difference is that the CAR is a continuous variable. Kinoshita *et al.* (*33*) believed that systems, which score the serum CRP and albumin levels separately may underestimate or overestimate the effects of CRP and albumin, so their predictive ability may not be as good as CAR. As demonstrated in our study, an increased CAR and a higher score for GPS, mGPS and HS-mGPS were all associated with higher tumor malignancy and shorter OS. However, according to the ROC analysis, the AUC values of CAR are significantly higher than the GPS, mGPS and HS-mGPS in predicting the 1-year and 3-year overall survival. The result indicates that the predicted value of CAR is more accurate and reliable than the other three indicators.

It was previously reported that a high serum level of CA 19-9 was an independent risk factor for poor prognosis in patients with GBC (*34*). In this research, although univariate analysis verified that CA 19-9 was a risk factor for poor prognosis, the result of multivariate analysis showed that the independent risk factors for poor prognosis of GBC patients were high CAR, poor tumor differentiation, advanced T stage and postoperative complications, rather than CA 19-9 (In this study, we didn't include the TNM stage into the multivariate analysis because it is colinear with T stage and N stage). Considering that tumor stage and differentiation need to be evaluated postoperatively, CAR can be easily measured preoperatively, and the price is much lower than CA 19-9. Therefore, it can be adopted as an effective tool for predicting the GBC prognosis before surgery.

However, there are still some limitations in our study. First of all, this is a single-center retrospective study with a small sample size, and insufficient sample size may lead to selection bias. Secondly, the subjects of this study are patients undergoing radical surgery, and only a small proportion of patients had preoperative jaundice. Therefore, we did not analyze the relationship between those indicators and preoperative jaundice. Third, since this study is restricted to Asians, the results may not apply to other races. Hence, a multi-center

prospective study is necessary to validate our results.

In conclusion, our study demonstrated that the CAR is the best prognostic predictor among the CRP-albumin-related markers for GBC patients. It's not only associated with tumor progression but also is an independent risk factors for poor prognosis.

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*Address correspondence to:
Donglin Sun and Weibo Chen, Department of Hepatopancreatobiliary Surgery, The Third Affiliated Hospital of Soochow University, 185 Juqian Street, Changzhou, Jiangsu 213003, China.
E-mail: czyysdl@163.com (Sun DL), cwb_med@163.com (Chen WB)

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Original Article

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Selection of patients with esophageal varices for liver resection of hepatocellular carcinoma

Yutaka Midorikawa[1,*], Tadatoshi Takayama[1], Tokio Higaki[1], Osamu Aramaki[1], Nao Yoshida[1], Kenichi Teramoto[1], Shingo Tsuji[2]

[1] Department of Digestive Surgery, Nihon University School of Medicine, Tokyo, Japan;
[2] Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan.

SUMMARY The presence of esophageal varices (EV) is a phenotype of portal hypertension, and the indications of liver resection for hepatocellular carcinoma (HCC) in patients with concomitant EV are conflicting. This retrospective study aimed to elucidate if there is justification for liver resection in patients with EV. The surgical outcomes were compared between the patients who underwent resection for HCC with EV (EV group) and those without EV (non-EV group) after propensity-score matching. More bleeding was prevalent ($P < 0.001$) and refractory ascites was more frequently observed ($P = 0.031$) in the EV group ($n = 277$) compared with the non-EV group ($n = 277$); however, the numbers of patients with morbidities ($P = 0.740$) and re-operation ($P = 0.235$) were not significantly different between the two groups. After a median follow-up period of 3.0 years, the median overall and recurrence-free survival periods of patients with EV were 4.8 years (95% confidence interval [CI], 4.1-5.9) and 1.7 years (1.5-2.0), respectively, and were significantly shorter than those of patients without EV (7.6 years [95% CI, 6.3.9.7], $P < 0.001$, and 2.2 years [1.9-2.5], $P = 0.016$). On multivariate analysis, the independent factors for overall survival in the EV group were indocyanine green clearance rate at 15 minutes, des-gamma carboxyprothrombin, and the presence of multiple tumors. Considering that liver resection for patients with EV can be safely performed, it should not be contraindicated. However, surgical outcomes of these patients were unsatisfactory, suggesting that candidates for resection for HCC should be carefully selected.

Keywords liver resection, esophageal varices, hepatocellular carcinoma

1. Introduction

The presence of esophageal varices (EV) is a phenotype of portal hypertension (*1*). The Barcelona Clinic Liver Cancer (BCLC) staging system proposed that liver resection is recommended only for Child-Pugh class A and B patients with a single hepatocellular carcinoma (HCC); additionally, those with portal hypertension are not candidates for operation due to the high frequency of postoperative mortality and worse patient survival (*2*). In contrast, as long as EVs are properly managed, based on the endoscopic findings before surgery, the presence of EV is not included in the exclusion criteria for resection in the treatment algorithms, according to the Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan (*3*).

The indications for liver resection in patients with HCC and EV are conflicting. Consistent with the BCLC staging system, previous reports demonstrated that patients with portal hypertension were not candidates for liver resection due to concomitant cirrhosis and thrombocytopenia, especially in Western countries (*4,5*). On the other hand, recent studies have reported that the presence of EV (*6-8*) or portal hypertension (*9-12*) is not an absolute contraindication for liver resection because the surgical outcomes of patients with HCC who underwent appropriate management for EV were acceptable. Thus, the outcomes of surgical resection for HCC in patients with portal hypertension are not completely understood.

To elucidate if there is justification for liver resection for HCC in patients with EV, we compared the surgical outcomes of patients with HCC who had EV to those of patients who did not have EV, and estimated the safety of operation and prognosis in these patients.

2. Patients and Methods

2.1. Patients

The study group was comprised of patients who

underwent liver resection for HCC at Nihon University Itabashi Hospital between 2000 and 2018. Among these patients, those who underwent initial and curative resection were included in this study. All patients were closely observed during each outpatient office visit after the operation. Each participant provided written, informed consent, and this study was approved by the institutional review board of Nihon University (RK-200908-8). The study design was conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

2.2. Esophageal varices

Upper gastrointestinal endoscopy was performed as previously described (*13*). Briefly, endoscopy was performed by two operators with expertise in the assessment of patients before surgery. Subsequently, patients with gastric or esophageal varices were defined as the EV group, while those without varices were part of the non-EV group. Surgical outcomes between the two groups were compared after propensity-score matching to adjust for patient background information, including age, sex, hepatitis viral infection, alcohol abuse, diabetes mellitus, tumor status, and tumor markers. Propensity scores were matched using a caliper width of 0.2 and one-to-one pair matching was carried out.

EVs were staged as none (no veins above the esophageal mucosal surface; F0), small (minimally elevated veins above the esophageal mucosal surface; F1), medium (tortuous veins occupying less than one-third of the esophageal lumen; F2), or large (those occupying more than one-third of the esophageal lumen; F3), according to the General Rules for Recording Endoscopic Findings of Esophagogastric Varices based on Beppu's classification (*14*). Interventional treatments, such as endoscopic variceal ligation, are required for severe EVs (F2/F3 EVs or F1 EVs with red-color signs) before surgery (*13*).

2.3. Surgical procedure

All patients underwent liver resection *via* an open approach. The indications for liver resection were determined based on the Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan (*3*), and the surgical procedure was determined by assessing the liver functional reserve, including the total serum bilirubin level and indocyanine green clearance rate at 15 minutes (ICGR15) (*15*). The liver was transected under ultrasonographic guidance using the clamp-crushing method with the inflow-blood-occlusion technique (*16*). Curative resection was defined as the complete removal of recognizable HCC with macroscopically tumor-free surgical margins. Anatomical resection was defined as liver resection over subsegmentectomy, and major resection included segmentectomy, hemihepatectomy, and trisegmentectomy.

2.4. Follow-up after surgery

All patients were followed for postoperative recurrence, as described previously (*17*). Briefly, tumor marker levels, such as alpha-fetoprotein (AFP) and des-gamma carboxyprothrombin (DCP) were measured, and imaging modalities, including computed tomography and ultrasonography, were performed every three months in all patients. Recurrence was diagnosed by dynamic computed tomography and/or gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging. The date of recurrence was defined as the date of examination when HCC recurrence was noted. In patients with recurrent HCC, the recurrence-free period was defined as the time between the date of surgery and the date of recurrence. Recurrent HCC was managed aggressively by performing repeated liver resection, transcatheter arterial chemoembolization, radiofrequency ablation, and chemotherapy according to the status of the HCC and liver function at the time of recurrence.

2.5. Complications

Complications specific to liver resection were defined as described previously (*18*). Morbidities were defined as complications with Clavien-Dindo classification grade ≥ 3b (*19*). In-hospital death was defined as the state of death within 90 days after liver resection.

2.6. Statistical analyses

Data collected from each group were statistically analyzed using Fisher's exact test and Wilcoxon rank-sum test, as appropriate. Survival rates were calculated using the Kaplan-Meier method and subsequently compared using the log-rank test. Prognostic factors for overall survival were identified using the Cox proportional hazards regression model. A P-value < 0.10 was set as the cutoff value for elimination. The following 17 variables, which were considered potential confounders, were examined: age (≥ *vs*. < 70 years), sex, positive for hepatitis B virus and C virus infection, alcohol abuse, diabetes mellitus, Child-Pugh classification (5 *vs*. 6 or 7), platelet count (≥ *vs*. $10 \times 10^4/\mu L$), ICGR15 (≥ *vs*. < 15%), serum AFP level (≥ *vs*. < 100 ng/mL), serum DCP level (≥ *vs*. < 100 ng/mL), and pathological findings of the main tumor [maximal tumor size [≥ *vs*. < 3.0 cm], multiple tumors (solitary *vs*. tumor number ≥ 2], tumor thrombus, tumor differentiation grade [poorly *vs*. well or moderately], surgical margin, and liver cirrhosis). All statistical analyses were performed using JMP version 12.0.1 (SAS Institute, Cary, NC, USA) and R version 3.4.0 (The R Foundation for Statistical Computing,

Vienna, Austria). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Patients

A total of 1,302 patients underwent initial and curative resection for HCC; they were divided into patients with gastric or esophageal varices (EV group, $n = 277$) and those without varices (non-EV group, $n = 1,025$) by upper gastrointestinal endoscopy before surgery (Figure 1). Before propensity-score matching, the liver function was worse, and hepatitis C virus infection ($P < 0.001$) and liver cirrhosis were more frequent ($P < 0.001$) in the EV group (Tables 1 and 2). In contrast, tumor status was more advanced, and AFP levels were lower ($P = 0.041$), while the DCP level was higher ($P = 0.004$) in the non-EV group. In the EV group, 56 patients (20.2%) were diagnosed with severe EV and underwent endoscopic variceal ligation.

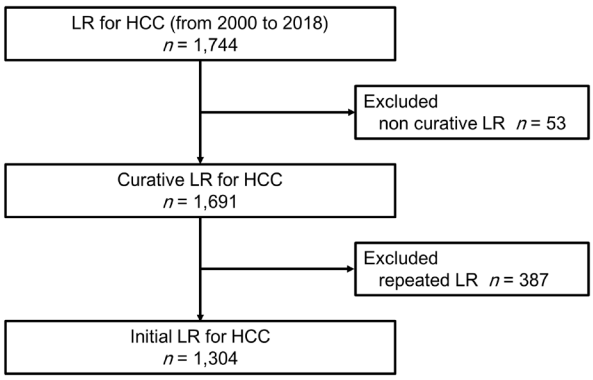

Figure 1. Flow diagram of patient recruitment and follow-up. LR, liver resection; HCC, hepatocellular carcinoma

3.2. Operative procedure

After one-to-one propensity matching, patients in the non-EV group underwent anatomic resection ($P = 0.002$) and major liver resection ($P = 0.003$) more frequently compared with those in the EV group (Table 3). The amount of bleeding was higher ($P < 0.001$) and the overall complication rate was more frequent ($P = 0.005$) in the EV group; however, the morbidity rate (Clavien-Dindo classification grade ≥ 3b) was not different between the two groups ($P = 0.740$). Regarding complications specific to liver resection for HCC, there

Table 1. Patient background

Items	Before propensity-score matching			After propensity-score matching		
	EV (+) ($n = 277$)	EV (−) ($n = 1,025$)	*P*	EV (+) ($n = 277$)	EV (−) ($n = 277$)	*P*
Age, years	68 (36-85)	69 (32-86)	0.093	68 (36-85)	68 (35-85)	0.774
Sex, male	201 (72.5)	792 (77.2)	0.111	201 (72.5)	202 (27.0)	1
Hepatitis B	42 (15.1)	169 (16.4)	0.646	42 (15.1)	52 (18.7)	0.308
Hepatitis C	173 (62.4)	569 (45.7)	< 0.001	173 (62.4)	161 (58.1)	0.339
Alcoholic	76 (27.4)	273 (26.6)	0.818	76 (27.4)	79 (28.5)	0.849
Diabetes mellitus	79 (28.5)	342 (33.3)	0.129	79 (28.5)	73 (26.3)	0.634
Child-Pugh, ≥ 6	96 (34.6)	196 (19.1)	< 0.001	96 (34.6)	54 (19.4)	< 0.001
Platelet, × 10^4/μL	10.4 (3.8-66.0)	15.8 (2.4-68.6)	< 0.001	10.4 (3.8-66.0)	14.7 (2.4-51.0)	< 0.001
ICGR15, %	17.8 (2.0-65.5)	11.6 (1.3-56.4)	< 0.001	17.8 (2.0-65.5)	12.1 (1.9-56.4)	< 0.001
Alpha-fetoprotein, ng/mL	19 (1-39,596)	12 (1-541,432)	0.041	19 (1-39,596)	18 (1-11,927)	0.751
DCP, mAU/mL	50 (1-75,000)	70 (1-75,000)	0.004	50 (1-75,000)	46 (6-21,851)	0.869

Data are presented as median (range) or *n* (%). ICGR15, indocyanine green clearance rate at 15 minutes; DCP, des-gamma-carboxy prothrombin.

Table 2. Pathology

Items	Before propensity-score matching			After propensity-score matching		
	EV (+) ($n = 277$)	EV (−) ($n = 1,025$)	*P*	EV (+) ($n = 277$)	EV (−) ($n = 277$)	*P*
Tumor size, cm	2.8 (0.7-13.0)	3.4 (0.5-21.0)	< 0.001	2.8 (0.7-13.0)	2.9 (0.5-16.0)	0.948
Multiple	72 (25.9)	254 (24.7)	0.696	72 (25.9)	76 (27.4)	0.773
Vascular invasion	64 (23.1)	319 (31.1)	0.009	64 (23.1)	64 (23.1)	1
Differentiation grade, por	25 (9.0)	114 (11.1)	0.380	25 (9.0)	27 (9.7)	0.884
Tumor exposure, positive	20 (7.2)	91 (8.8)	0.466	20 (7.2)	17 (6.1)	0.734
Cirrhosis	188 (67.8)	260 (25.3)	< 0.001	188 (67.8)	90 (32.4)	< 0.001

Data are presented as median (range) or *n* (%).

were two patients (0.3%) with liver failure only in the EV group (P = 0.499), and refractory ascites was more frequent in the EV group (17 patients [6.1%]) than in the non-EV group (six patients [2.1%]) (P = 0.031).

In this series, 17 (6.1%) patients in the EV group and 10 (3.6%) patients in the non-EV group underwent re-operation (P = 0.235) for severe postoperative complications, including intraperitoneal hemorrhage in 11 patients (1.9%), intra-peritoneal abscess in five patients (0.9%), bile leakage in four patients (0.7%), portal vein thrombus in two patients (0.3%), wound infection in two patients (0.3%), gastrointestinal perforation in two patients (0.3%), and ileus in one patient (0.2%). Two patients (0.3%) died after the operation; one died from rapture of the varices, while another died from liver failure in the EV group (P = 0.499).

Table 3. Operative data

Items	EV (+) (n = 277)	EV (−) (n = 277)	P
Operative time, min	312 (113-632)	300 (97-705)	0.337
Clamp time, min	66 (0-485)	66 (0-516)	0.606
Bleeding, mL	316 (5-3,887)	235 (10-3,500)	< 0.001
Transfusion	17 (6.1)	15 (5.4)	0.855
Anatomic resection	66 (23.8)	99 (35.7)	0.002
Major resection	13 (4.6)	33 (11.9)	0.003
Complications			
Overall†	131 (47.2)	98 (35.3)	0.005
Liver failure	2 (0.7)	0	0.499
Ascites	17 (6.1)	6 (2.1)	0.031
Morbidities	21 (5.7)	18 (4.3)	0.740
Re-operation	17 (6.1)	10 (3.6)	0.235
Intra-peritoneal hemorrhage	6 (2.1)	5 (1.8)	1
Intra-peritoneal abscess	4 (1.4)	1 (0.3)	0.372
Bile leakage	2 (0.7)	2 (0.7)	1
Portal venous thrombus	2 (0.7)	0	0.499
Others	3 (1.0)	2 (0.7)	1
Operative death	2 (0.7)	0	0.499
Hospital stay, days	14 (8-190)	13 (5-81)	0.112

Data are presented as median (range) or n (%). †, Only complications specific to liver resection were enumerated.

3.3. Survival

After a median follow-up of 3.0 years (range, 0.2-16.3), a total of 345 patients (62.2%) had recurrence: 325 patients (94.2%) experienced recurrence in the remnant liver, 11 patients (3.1%) experienced recurrence in the distant sites including the lung, lymph nodes, peritoneum, and bone, and 9 patients (2.6%) had both intra- and extrahepatic recurrence (Table 4). Resection for recurrent HCC was performed more frequently in the non-EV group (P = 0.087).

The median overall and recurrence-free survival periods in the EV group (n = 277) were 4.8 years (95% confidence interval [CI], 4.1-5.9) and 1.7 years (1.5-2.0), respectively, and were significantly shorter compared with those in the non-EV group (n = 277) (7.6 years [95% CI, 6.3-9.7], P < 0.001, and 2.2 years [1.9-2.5], P = 0.016) (Figure 2). The 5-year overall survival rates were 48.9% and 66.5%, and the 5-year recurrence-free survival rates were 19.6% and 27.4% in the EV and non-EV groups, respectively. On multivariate analysis, the independent factors for overall survival

Table 4. Treatment for recurrence

Items	EV (+) (n = 189)	EV (−) (n = 156)	P
Recurrent sites			1
Intrahepatic	178 (94.1)	147 (94.2)	
Distant sites	6 (3.1)	5 (3.2)	
Both	5 (2.6)	4 (2.5)	
Treatments			0.093
Second resection	56 (29.6)	60 (38.4)	
TACE/TAI	118 (62.4)	83 (53.2)	
RFA	2 (1.0)	4 (2.5)	
Chemotherapy	5 (2.6)	8 (5.1)	
Radiation therapy	2 (1.0)	0	
None	5 (2.6)	1 (0.6)	

Data are presented as n (%). TACE, transcatheter arterial chemoembolization; TAI, transcatheter arterial infusion; RFA, radiofrequency ablation.

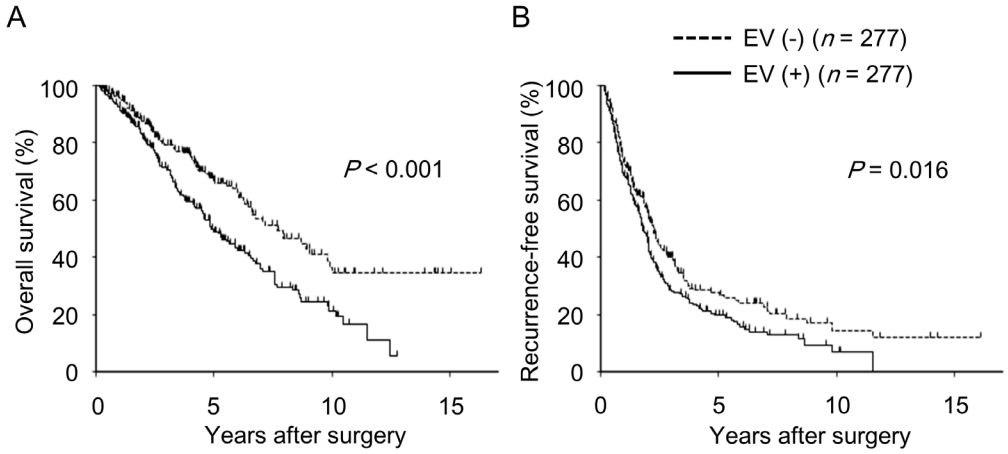

Figure 2. Overall survival and recurrence-free survival of patients with HCC. There were significant differences between patients with EV and those without EV in overall survival **(A)** and recurrence-free survival **(B)**. EV, esophageal varices; HCC, hepatocellular carcinoma

in the EV group were ICGR15 (P = 0.019), DCP (P = 0.002), and multiple tumors (P = 0.016) (Table 5). The median overall survival times were significantly shorter in patients with ICGR15 ≥ 15% (4.6 years [range, 4.1-5.7 years] versus 7.5 years [6.5-9.8 years], P < 0.001), those with DCP ≥ 100 mAU/mL (4.5 years [range, 3.5-6.0 years] versus 6.9 years [6.0-7.6 years], P < 0.001), and those with multiple tumors (4.3 years [range, 3.4-5.7 years] versus 6.7 years [6.0-7.8 years], P < 0.001) in the EV group (Figure 3).

4. Discussion

Our study demonstrated that liver resection for patients with gastric or esophageal varices can be safely performed. However, survival in the EV group was

Table 5. Prognostic factors for survival of patients with EV

Items	Univariate			Multivariate		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Age	1.19	0.85-1.67	0.296			
Sex	0.89	0.62-1.29	0.541			
Hepatitis B	1.04	0.63-1.64	0.847			
Hepatitis C	0.90	0.63-1.30	0.586			
Alcohol	0.90	0.60-1.30	0.594			
Diabetes mellitus	0.82	0.54-1.23	0.363			
Child-Pugh	1.33	0.95-1.86	0.090	1.08	0.76-1.54	*0.545*
Platelet	1.02	0.73-1.43	0.864			
ICGR15	1.57	1.10-2.27	0.011	1.54	1.07-2.26	*0.019*
Alpha-fetoprotein	1.15	0.78-1.66	0.467			
DCP	2.03	1.43-2.85	< 0.001	1.75	1.21-2.52	*0.002*
Tumor size	1.57	1.12-2.20	0.008	1.40	0.98-1.99	*0.059*
Multiple tumor	1.67	1.16-2.37	0.006	1.60	1.09-2.33	*0.016*
Tumor thrombus	1.28	0.86-1.86	0.213			
Differentiation grade	1.02	0.53-1.76	0.947			
Surgical margin	0.94	0.44-1.74	0.858			
Cirrhosis	1.38	0.96-2.04	0.079	1.22	0.82-1.84	*0.327*

EV, esophageal varices; ICGR15, indocyanine green clearance rate at 15 minutes; DCP, des-gamma-carboxy prothrombin.

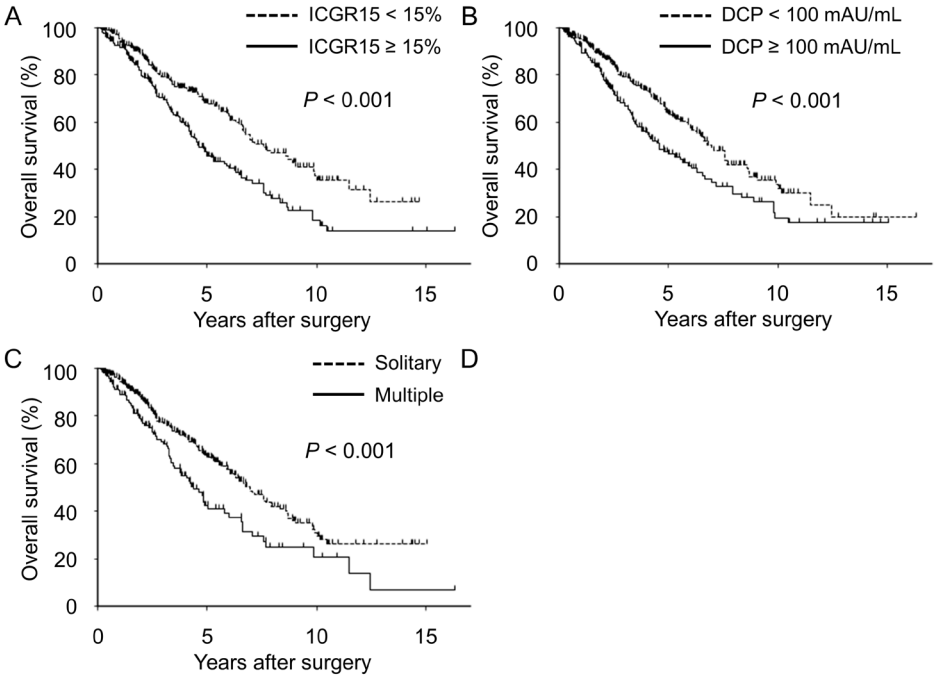

Figure 3. Overall survivals of patients with HCC in the EV group. (A) The median overall survival of patients with ICGR15 ≥ 15% (n =170) was significantly shorter than those with ICGR15 < 15% (n =107) (P < 0.001). **(B)** The median overall survival of patients with DCP ≥ 100 mAU/mL (n =97) was significantly shorter than those with DCP < 100 mAU/mL (n = 180) (P < 0.001). **(C)** The median overall survival of patients with multiple tumor (n =72) was significantly shorter than those with solitary tumor (n =205) (P < 0.001). HCC, hepatocellular carcinoma; EV, esophageal varices; ICGR15, indocyanine green clearance rate at 15 minutes; DCP, des-gamma-carboxy prothrombin.

significantly worse than that in the non-EV group; therefore, indications for liver resection for such patients should be carefully determined.

Liver cirrhosis was more frequent and liver function was poorer in the EV group than in the non-EV group in this study. Consistent with a previous report (*20*), the amount of bleeding was larger in the EV group, despite the fact that major resection was less frequent. Considering that the augmentation of blood loss increases postoperative complications in resection for HCC (*21*), overall complication rate was more frequent in the EV group in this study. In particular, the presence of refractory ascites was more frequent in the EV group (*22,23*). However, the frequency of severe complications, such as liver failure, did not differ between the two groups. Re-operation was often performed for intra-peritoneal hemorrhage, intra-peritoneal abscess, and bile leakage, but its frequency also was not significantly different between the two groups. Because perioperative prophylactic management of gastric or esophageal varices is effective for preventing rupture after surgery (*11,13*), we supposed that liver resection for HCC in patients with varices could be safely performed and should not be contraindicated (*6,8,24*).

Due to poor liver function, anatomical resection and major resection were less frequent in the EV group (*15*); this could negatively affect the survival of patients undergoing non-anatomical resection (*25*). Furthermore, liver function, which is one of the most influential predictors of survival for patients with HCC, was worse (*26,27*). Therefore, consistent with a previous study (*6*), both overall and recurrence-free survival were significantly shorter in the EV group. Nevertheless, it has been shown that patients with HCC with EVs are no longer a contraindication to liver resection because the presence of EV is not always a negative predictor for survival of patients (*6,8,24*). Resection could even provide a survival benefit for patients with portal hypertension and a history of Child-Pugh class A cirrhosis (*10*). Actually, unlike in the BCLC treatment strategy, portal hypertension is not a contraindication for liver resection in the Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan (*3*).

Multivariate analysis showed that ICGR15, DCP, and multiple tumors were independent factors for overall survival (*28,29*). Certainly, liver resection for HCC patients with EV can be safely performed and it may provide survival benefits for such patients. However, the prognosis of patients with unfavorable liver function and advanced HCC is extremely poor. Therefore, indications for liver resection for patients with such factors and EV should be carefully determined.

This study has several limitations. First, the most appropriate control group in this study included patients undergoing other treatments such as transcatheter arterial chemoembolization (*30*) or radiofrequency ablation (*31*). Survival benefits of liver resection could have been revealed for the first time by comparing the survival rates of patients undergoing surgery with those of patients undergoing other treatments. However, this study was based on the patient records of the surgical department; therefore, we have no data for patients with EV who underwent other treatments. Second, this retrospective study was likely affected by selection bias, especially in the EV group. Generally, the liver function of patients with HCC was worse, and patients with poorer general conditions, which did not appear as abnormal laboratory data, might have been excluded.

In conclusion, the survival of patients with gastric or esophageal varices was significantly shorter than that of patients without varices. However, liver resection of such patients could be safely performed and should not be contraindicated. On the other hand, the prognosis of patients with EV who had unfavorable liver function and/or advanced tumor was unsatisfactory; therefore, candidates for surgery for such patients should be carefully selected based on their liver function and tumor status.

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**Address correspondence to:*
Yutaka Midorikawa, Department of Digestive Surgery, Nihon University School of Medicine, 30-1, Oyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan.
E-mail: mido-tky@umin.ac.jp

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Original Article

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Risk factors for hepatitis B virus recurrence after living donor liver transplantation: A 22-year experience at a single center

Sung Kwan Bae[1], Nobuhisa Akamatsu[1,2], Akihiko Ichida[2], Harufumi Maki[2], Yujiro Nishioka[2], Takuya Kawahara[3], Mayumi Hoshikawa[2], Rihito Nagata[2], Yuichiro Mihara[2], Yoshikuni Kawaguchi[2], Takeaki Ishizawa[2], Junichi Arita[2], Junichi Kaneko[2], Sumihito Tamura[2], Kiyoshi Hasegawa[1,2,*]

[1] Organ Transplantation Service, The University of Tokyo Hospital, Tokyo, Japan;
[2] Artificial Organ and Transplantation Division, Department of Surgery, The University of Tokyo, Tokyo, Japan;
[3] Biostatistics Division, Clinical Research Support Center, The University of Tokyo Hospital, Tokyo, Japan.

SUMMARY The factors associated with hepatitis B virus (HBV) recurrence after living donor liver transplantation (LDLT) have not been fully clarified. The aim of this study was to determine the risk factors associated with HBV recurrence after LDLT. From January 1996 to December 2018, a total of 609 LDLT operations were performed at our center. A retrospective review was performed of 70 patients (male, $n = 59$; female, $n = 11$; median age = 54 years) who underwent LDLT for HBV-related liver disease. The virologic and biochemical data, tumor burden, antiviral and immunosuppressive therapy were evaluated and compared between the HBV recurrence and non-recurrence groups. Eleven of 70 patients (16%) developed post-LDLT HBV recurrence. The overall actuarial rates of HBV recurrence at 1, 3, 5, 10, and 20 years were 0%, 13%, 16.7%, 18.8%, and 18.8%, respectively. The median interval between LDLT and HBV recurrence was 57 months (range, 18-124 months). Based on the univariate and multivariate analyses, a serum HBV DNA level of ≥ 4 log copies/mL (hazard ratio [HR], 4.861; 95% confidence interval [95% CI], 1.172-20.165; $P = 0.029$), and hepatocellular carcinoma (HCC) beyond the Milan criteria (HR, 10.083; 95% CI, 2.749-36.982; $P < 0.001$) were independent risk factors for HBV recurrence after LDLT. In LDLT patients, high pre-LT HBV DNA levels and HCC beyond the Milan criteria were risk factors for HBV recurrence. With the current expansion of the LT criteria for HCC, we should remain cautious regarding the risk of HBV recurrence, particularly in these groups.

Keywords living donor liver transplantation, HBV recurrence, HBV DNA, HCC, Milan criteria

1. Introduction

Hepatitis B is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) worldwide. In the era without effective prophylaxis, hepatitis B virus (HBV)-related disease was a relative contraindication for liver transplantation (LT) until the mid-1990s (*1*). Since the mid-1990s, several prophylaxis strategies for recurrent HBV have shown great progress (*2*), with the use of anti-hepatitis B immunoglobulin (HBIG) and nucleos(t)ide analogues (NAs). However, approximately 10% of transplanted patients still experience HBV recurrence (*3,4*). In previous studies, the factors associated with HBV recurrence were reported to include non-fulminant hepatitis B (*5*), a high pre-LT HBV DNA level (*6,7*), hepatitis B e antigen (HBeAg) positivity (*8*), immunosuppression from steroids and systemic chemotherapy (9), and pre-LT HCC and post-LT HCC recurrence (*10-13*).

The aim of the present study was to assess the incidence and risk factors associated with HBV recurrence in living donor liver transplantation (LDLT) recipients over a 20-year period. We analyzed a retrospective series of patients who underwent LDLT for HBV-related liver disease, and evaluated their virologic and biochemical data, tumor burden, antiviral therapy, and immunosuppressive therapy.

2. Methods

2.1. Patients

From January 1996 to December 2018, a total of 609 LDLT procedures were performed at The University

of Tokyo Hospital. We retrospectively reviewed all demographic, radiologic and laboratory data, which were recorded in a computerized database in the study period. Among these patients, 70 (12%) patients underwent LDLT for HBV-related liver disease, and were enrolled in this study. Patient data were censored at death or at the time of the last follow-up examination.

The study protocol complied with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki and was approved by the Research Ethics Committee/ Institutional Review Board of the Graduate School of Medicine and Faculty of Medicine, The University of Tokyo (project number 2158).

2.2. Immunoprophylaxis

Prior to 2008, prophylactic post-transplant treatment was based on HBIG (Hebsbulin-IH; Japan Blood Products Organization, Tokyo, Japan) monotherapy. After 2008, all patients were treated with a combination of HBIG and at least one NA agent (lamivudine, adefovir, entecavir, tenofovir or a combination thereof) for HBV prophylaxis after transplantation. HBIG (10,000 IU, intravenous) was administered during the anhepatic phase and just after the end of the operation. Thereafter, HBIG was administered to maintain hepatitis B surface antibody (anti-HBs) levels at > 1,000 IU/L for 3 months and > 500 IU/L within 1 year; finally, the HBsAb titer was maintained at 100-200 IU/L for > 1 year after transplantation.

2.3. Immunosuppression protocol

The details of the immunosuppression protocol are described elsewhere (*14*). The post-transplant immunosuppression regimen consisted of a steroid and a calcineurin inhibitor, such as tacrolimus or cyclosporine, with or without mycophenolate mofetil. The immunosuppressive dosing was adjusted according to the therapeutic drug levels and the renal function. Maintenance tacrolimus therapy after 6 months was targeted at a level of 5 ng/mL, and a cyclosporine trough level of 100 ng/mL was maintained, depending on the rejection profile. The monitoring of trough serum levels was conducted regularly as per protocol for the evaluation of toxicity and compliance and for dose adjustments. Methylprednisolone was prescribed at a dose of 0.05 mg/kg more than 6 months after LT.

2.4. Serological monitoring

HBV recurrence was defined as the development of hepatitis B surface antigen (HBsAg) positivity and/or HBV DNA positivity after LT (*15*). Standard biochemical tests of the liver function were performed at each follow-up visit. The measurement of HBsAg and anti-HBs was carried out in the University of Tokyo Hospital using commercial chemiluminescent immunoassay (CLIA) kits in an ARCHITECT ANALYSER i2000 (Abbott Japan Co., Ltd., Tokyo, Japan). The sensitivity of the HBsAg assay ranged from 0.05 to 250 IU/mL. Specimens with an HBsAg value of > 250 IU/mL were diluted to 1:500 using a diluent recommended by the manufacturer. The exact concentrations of the samples have been measured since 2014. The sensitivity of the anti-HBs assay ranged from 6.0 to 1000 mIU/mL. The HBV DNA levels were quantified with a transcription-mediated amplification assay (Mitsubishi Chemical Medience, Tokyo, Japan), which has a detection range of 3.7-8.7 log genome equivalents (LGE)/mL, until March 2004. Thereafter, all HBV DNA levels were quantified using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6 to 7.6 log copies/ml, or a COBAS TaqMan HBV Test v2.0 (Roche Diagnostics), which has a dynamic range of 2.1 to 9.0 log copies/mL (1.3 to 8.2 log IU/mL).

2.5. Vaccination

Among the subjects of the study, 28 patients were vaccinated in accordance with the one-year HBV vaccination protocol (*16*). After completion of the one-year vaccination protocol, patients were followed for an additional two years, with monthly measurements of the HBsAb titer and records of the required dose of HBIG for each patient in order to clarify the long-term efficacy of vaccination.

2.6. Statistical analyses

We assessed the cumulative incidence of HBV recurrence after LDLT and overall survival with a Kaplan-Meier curve analysis. We calculated the hazard ratios (HRs) for the time to HBV recurrence with a Cox proportional hazards model using each potential predictor as a covariate. *P* values of < 0.05 were considered to indicate statistical significance, and *p* values of < 0.15 were considered to indicate candidate potential predictors. All statistical analyses were performed using the SPSS statistics version 23.0 software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Patient demographics

The patient characteristics are shown in Table 1. The population included 59 men and 11 women, with median age of 54 years (range, 38-67 years). The median follow-up period after LT was 134 months (range, 1-226 months). HCC was present in 39 patients (56%). Among them, 37 patients with HCC were diagnosed by preoperative computed tomography or magnetic resonance imaging, whereas two patients were diagnosed incidentally by a pathological examination of

the explant. According to the explant pathology, HCC beyond the Milan criteria (no macrovascular invasion, a single tumor ≤ 5 cm, or up to three tumors ≤ 3 cm each) was present in 16 patients (41%). Pre-LT HCC therapy was administered to 28 of the 39 patients with HCC (72%). Among them, 23 patients (64%) received a combination of local ablative therapy in the form of arterial chemoembolization, radiofrequency ablation and/or an ethanol injection prior to LDLT. Four patients (10%) underwent surgical resection of the tumor, and one patient (3%) received systemic chemotherapy prior to LDLT.

Table 1. The characteristics of the 70 patients with hepatitis B virus-related liver disease

Variable	
Age	54 (38-67)
Male/Female	59/11 (84%/16%)
HBsAg, +/-	69/1 (98%/2%)
HBeAg, +/-	14/56 (20%/80%)
HBV DNA (log copies/ml)	
< 2.6	49 (70%)
2.6-4.0	12 (17%)
> 4.0	9 (13%)
HCC, +/-	39/31(56%/44%)
Milan criteria, within/beyond	23/16 (59%/41%), *n* = 39
Pre-LT treatment of HCC	
Resection/TACE/TACE+RFA or PEIT/RFA/PEIT/TAI/none	7/18/9/1/1/1/33
Pre-LT antiviral therapy	
LMV/LMV +ADV/TDF/ETV/None	39/4/1/15/11
HBV Prophylaxis	
HBIG/HBIG+NA	52/18 (74%/26%)
LMV/LMV+ADV/ETV	1/3/14 (5%/17%/78%), *n* = 18
Follow-up period (months)	134 (1-226)

Qualitative variables are expressed as the number of patients with the percentage in parentheses, and quantitative variables are expressed as the median, with the range in parentheses. ADV, adefovir; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBIG, hepatitis B immunoglobulin; HBsAg, Hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LAM, lamivudine; NA, nucleoside analogue; LT, liver transplantation; PEIT, percutaneous ethanol injection therapy; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; TAI, transcatheter arterial infusion; TDF, tenofovir.

3.2. Risk factors for HBV recurrence after LDLT

Eleven of the 70 patients (16%) developed post-LT HBV recurrence. The overall actuarial rates of HBV recurrence after LDLT at 1, 3, 5, 10, and 20 years were 0%, 13%, 16.7%, 18.8%, and 18.8%, respectively (Figure 1A). Table 2 shows the results of the univariate and multivariate analyses of risk factors associated with HBV recurrence after LDLT. The univariate analyses revealed that the HCC beyond the Milan criteria (hazard ratio [HR], 7.592; 95% confidence interval [95% CI], 2.217-25.991; $P = 0.001$) was significantly associated with HBV recurrence. According to the multivariate analysis, serum pre-LT HBV DNA ≥ 4 log copies/mL (HR, 4.861; 95%CI, 1.172-20.165; P=0.029) and HCC beyond the Milan criteria (HR, 10.083; 95% CI, 2.749-36.982; $P < 0.001$) were independent risk factors for HBV recurrence after LDLT.

3.3. Pretransplant HCC and HCC recurrence

The cumulative HBV recurrence rate after LDLT in patients with HCC was higher (with marginal significance) than that in patients without HCC ($P = 0.080$) (Figure 1B). Meanwhile, the cumulative HBV

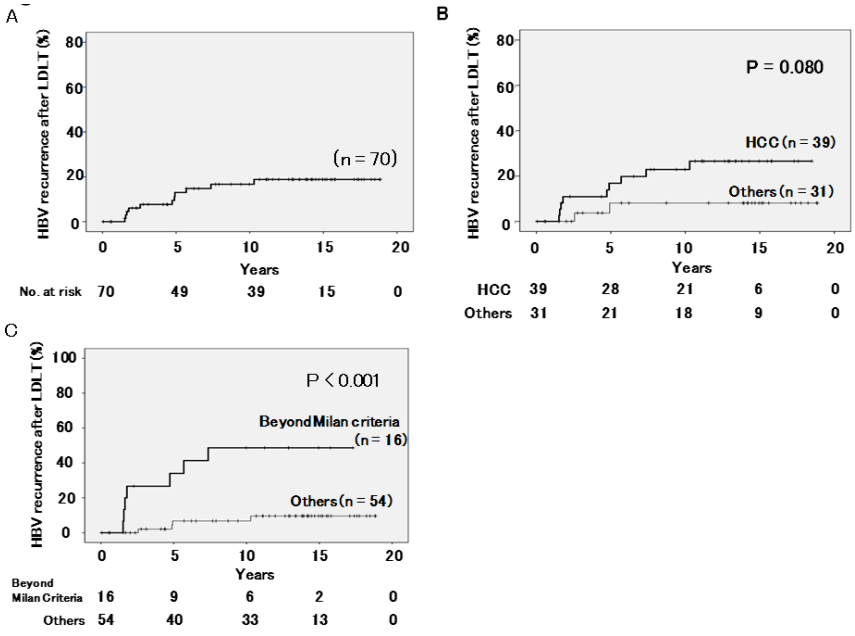

Figure 1. The cumulative rates of HBV recurrence after LDLT in recipients with HBV-related liver disease. (A) The overall analysis. **(B)** HCC versus others. **(C)** HCC beyond the Milan criteria versus others.

Table 2. Predictors of HBV recurrence after LT

Items	HBV recurrence (n = 11)	HBV non-recurrence (n = 59)	Univariate analysis			Multivariate analysis		
			HR	95% CI	P-value	HR	95% CI	P-value
Age, ≥ 55 (years)	7	32	1.416	0.414-4.839	0.579			
Sex, male	11	48	NE	NE	0.155			
HBeAg, +	3	11	1.462	0.387-5.517	0.575			
HBV DNA ≥ 4 (LC/mL)	3	6	2.702	0.716-10.203	0.143	4.861	1.172-20.165	0.029
Vaccination, +	3	25	0.350	0.092-1.337	0.125			
HCC, +	9	32	3.602	0.778-16.676	0.101			
Milan criteria, beyond	7	9	7.592	2.217-25.991	0.001	10.083	2.749-36.978	< 0.001
Pre-LT HCC treatment, +	7	30	1.516	0.444-5.181	0.507			
CNI, FK	9	47	1.222	0.264-5.658	0.797			
Prophylaxis, HBIG	10	42	2.684	0.341-21.141	0.348			

CI, confidence interval; CNI, calcineurin inhibitor; FK, tacrolimus; HBeAg, hepatitis B e-antigen; HBIG, hepatitis B immunoglobulin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; LC, log copies; LT, liver transplantation.

Table 3. Antiviral therapy administered and the outcomes of patients with HBV recurrence after LDLT

No.	Age (years), sex	HBeAg	HBVDNA (LC/mL)	Primary disease	Milan Criteria	HBV prophylaxis	HBV Recurrence (months)	HCC recurrence (months)	Outcome
1	56, M	-	ND	Cirrhosis	-	HBIG	59	-	Alive
2	39, M	+	4.0	Cirrhosis	-	HBIG	31	-	Alive
3	59, M	-	ND	HCC	Within	HBIG	124	-	Alive
4	48, M	+	4.9	HCC	Within	HBIG	59	-	Alive
5	55, M	-	ND	HCC	Beyond	HBIG	88	-	Alive
6	48, M	-	ND	HCC	Beyond	HBIG	68	-	Alive
7	52, M	+	5.2	HCC	Beyond	HBIG	57	-	Alive
8	53, M	-	3.7	HCC	Beyond	HBIG	20	14	Died
9	54, M	-	ND	HCC	Beyond	HBIG	18	19	Died
10	54, M	-	ND	HCC	Beyond	HBIG	18	20	Died
11	64, M	-	ND	HCC	Beyond	HBIG+ETV	21	22	Alive

ETV, entecavir; HBeAg, hepatitis B e-antigen; HBIG, hepatitis B immunoglobulin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LC, log copies; LDLT, living donor liver transplantation; ND, not detected.

recurrence rate in patients with HCC beyond the Milan criteria was significantly higher than that in those with HCC within the Milan criteria ($P < 0.001$) (Figure 1C). Among 16 patients with HCC beyond the Milan criteria, 7 patients (44%) developed HBV recurrence after LDLT (Table 3, case 5-11), and recurrent HCC developed in 4 of these 7 patients (57%) (case 8-11). In case 8, the tumors were beyond the Milan criteria at LDLT, and HCC recurred as lung metastasis at 14 months after transplantation. Despite the administration of chemotherapy, the HCC had grown, and HBV reappeared at 20 months after transplantation (Figure 2A). In case 9, there were uncountable HCC tumors, and the disease was beyond the Milan criteria at LDLT. At 18 months after transplantation, recurrent HBV was observed, and HCC recurred at 19 months after LDLT (Figure 2B). In case 10, there were 7 HCC tumors at LDLT. HBV reappeared at 18 months after transplantation, and HCC recurrence was observed as bone metastasis at 20 months after LDLT (Figure 2C). All 3 patients (cases 8-10) finally died of recurrent HCC, at 25, 33, 44 months after transplantation, respectively. Among the 4 patients with recurrent HCC, the remaining patient (case 11) was the only patient to survive. The tumors were beyond the Milan criteria at LDLT, and HBV reappeared at 21 months after LDLT, despite combined therapy with HBIG and entecavir. HCC recurred as lung metastasis at 22 months after transplantation, and lung resection was performed. HBsAg disappeared after the operation and the patient had an uneventful postoperative course (Figure 2D).

3.4. Vaccination

HBV vaccination was indicated for patients with normal or near normal liver function test results with a low level of immunosuppression, and the follow-up period after LDLT was at least one year (*16*). All 28 vaccinated patients received HBIG monoprophylaxis. Among them, 2 showed a good response to the vaccination with an increase in the anti-HBs titer, and HBIG was successfully discontinued. However, the remaining 26 patients were poor responders, including 3 who developed recurrent HBV after vaccination.

3.5. Overall survival after LDLT

The overall survival rates of the 70 recipients at 1, 3, 5,

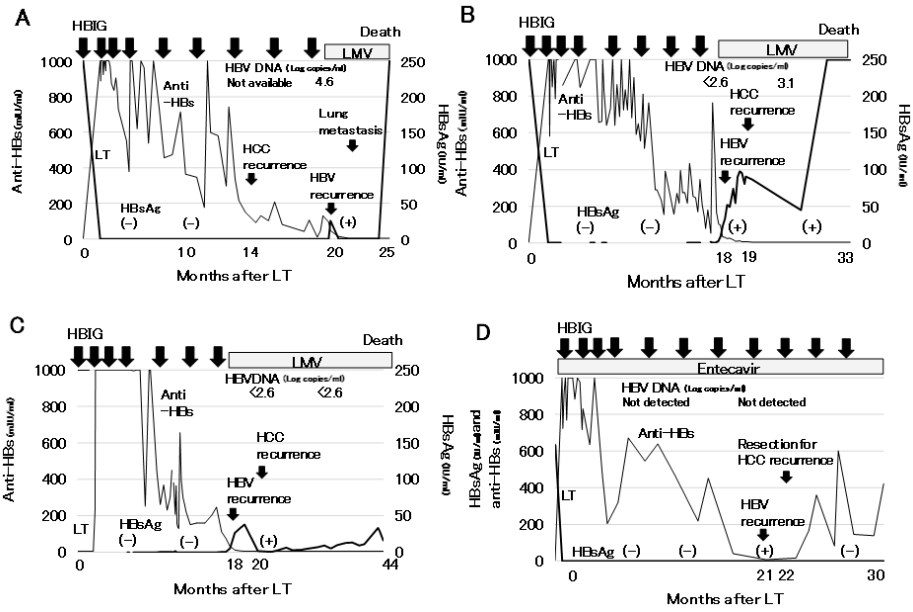

Figure 2. The clinical course of the patients with recurrent HBV after LDLT.

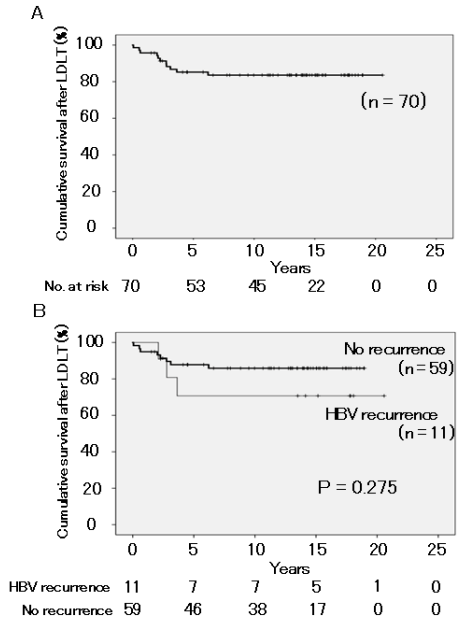

Figure 3. The cumulative rates of survival in LDLT recipients. (A) The overall analysis. **(B)** The patients with recurrent HBV versus those without.

10, and 20 years were 95.7%, 88.3%, 85.2%, 83.6%, and 83.6%, respectively (Figure 3A). During the study period, 11 recipients died: 5 from recurrent HCC, 2 from respiratory diseases, and the remaining 4 due to other reasons: graft failure due to hepatic vein stenosis, acute heart failure, cerebral hemorrhage, and colon cancer. The overall survival rate after LDLT was not significantly reduced in patients with recurrent HBV; the probability rates of survival at 1, 3, 5, 10, and 20 years were 100%, 80.8%, 70.7%, 70.7%, and 70.7%, respectively, in patients with recurrent HBV and 94.9%, 89.6%, 87.8%, 85.9%, and 85.9%, respectively, in patients without recurrent HBV ($P = 0.275$) (Figure 3B).

4. Discussion

In our study, we demonstrated that pre-LT HBV DNA ≥ 4 log copies/mL and HCC beyond the Milan criteria were independent risk factors for the recurrence of HBV after LDLT. A high pre-LT HBV DNA level is well known to be a factor associated with the recurrence of HBV after LT (*6,7*). In this respect, our results were consistent with previous reports.

Previous studies have suggested an association between HCC and a higher risk of HBV recurrence after transplantation (*10-13*). Faria *et al.* reported that the presence of HCC at transplantation and HCC recurrence were independent risk factors for HBV recurrence (*10*). Furthermore, Saab *et al.* reported that pre-LT HCC and the recurrence of HCC after transplantation were associated with HBV reinfection and with decreased patient survival (*11*). Several mechanisms may be involved in the higher rate of HBV recurrence in HCC patients. Although the immunosuppression related to HCC recurrence may itself contribute to HBV recurrence, the direct relationship between the resection of metastasis and the disappearance of HBsAg suggests HBV replication in HCC metastasis (Figure 2D). Faria *et al.* demonstrated the presence of covalently closed circular DNA in both HCC cells and in non-tumor cells in explanted livers, suggesting that HBV replication may also occur in tumor cells (*10*). Furthermore, Bai *et al.* indicated that cccDNA and pgRNA are detected and represented HBV replication not only in non-HCC

tissues but also in HCC tissues (*17*). In the current era of highly effective prophylaxis against HBV, these finding suggests that HCC itself is the main factor of HBV prophylaxis failure.

Since the Milan criteria were proposed in 1996, liver transplantation has become widely accepted for HCC patients with favorable tumor morphology. However, because of the strict limitations in patient selection, the Milan criteria were recently challenged by several studies to expand the patient selection (*18-22*). In Japan, expanded criteria for LT for HCC patients were recently proposed. Based on a data analysis of the Japanese nationwide survey, the 5-5-500 rule (nodule size ≤ 5 cm in diameter, nodule number ≤ 5, and alfa-fetoprotein value ≤ 500 ng/mL) were established as new expanded LT criteria for HCC patients (*23*). Meanwhile, in our study, among 16 patients with HCC beyond the Milan criteria, 7 patients developed recurrent HBV. Furthermore, HCC beyond the Milan criteria was an independent risk factor for the recurrence of HBV after LDLT. These results suggest that although it is crucial to widely accept HCC patients, the expansion of the Milan criteria is a double-edged sword with regard to the risk of HBV recurrence. Meanwhile, HCC recurrence occurred in 4 of these 7 patients with recurrent HBV (Table 2, case 8-11). It is noteworthy that HBV recurrence preceded HCC recurrence in 3 of 4 cases (Figure 2B, C, D). Especially in case 11, HCC recurrence was suspected at the time of HBV recurrence based on previous reports and our own experience, and resection for lung metastasis could be performed at an early stage (Figure 2D). Vatansever et al. reported that the HBV and HCC recurrence are closely related in patients who underwent LT due to HBV-associated HCC (*24*). They also insisted that the HBV recurrence after LT increases the risk of HCC recurrence. Therefore, they concluded that the HBV recurrence may be used as a predictor in forecasting the HCC recurrence. Whether HBV recurrence can be a definite predictor of the recurrence of HCC after LT is a matter of debate, however, these results should not be missed and require confirmation by further investigations.

The present study was associated with several limitations, including its retrospective design and relatively small sample size. However, to our knowledge, this is the first paper to call attention to the relationship between the expansion of the Milan criteria and HBV recurrence after LT. Furthermore, this study has the longest follow-up period among LDLT studies analyzing recipients with HBV-related liver disease.

In conclusion, HCC beyond the Milan criteria were independent risk factors for the recurrence of HBV in LDLT patients. In this era with the expansion of the LT criteria for HCC, we should remain cautious with regard to the risk of HBV recurrence, particularly in these groups. Further multicenter studies are needed in order to evaluate our results prospectively.

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*Address correspondence to:
Kiyoshi Hasegawa, Artificial Organ and Transplantation Division, Department of Surgery, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: kihase-tky@umin.ac.jp

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Brief Report

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Association of HIV infection with metabolic syndrome among normal or underweight young adults: evidence from the CHART cohort

Ruizi Shi[1,2,§], Xiaoxiao Chen[3,§], Haijiang Lin[1,3], Weiwei Shen[3], Xiaohui Xu[1], Bowen Zhu[1], Xiaoyi Xu[1], Yingying Ding[1], Frank Y. Wong[1,4,5,6], Na He[1,2,*]

[1] School of Public Health, Fudan University, Shanghai, China; and the Key Laboratory of Public Health Safety of Ministry of Education, Fudan University, Shanghai, China;
[2] Key Laboratory of Health Technology Assessment of Ministry of Health, Fudan University, Shanghai, China;
[3] Taizhou City Center for Disease Control and Prevention, Zhejiang Province, China;
[4] Center for Indigenous Nursing Research for Health Equity, Florida State University, Tallahassee, FL, U.S.A;
[5] Department of Psychology, College of Social Sciences, University of Hawai'i at Mānoa, Honolulu, HI, U.S.A;
[6] Department of Population Science, John D. Bower School of Population Health, University of Mississippi Medical Center, MS, U.S.A.

SUMMARY Metabolic syndrome (MS) is common among obese people. Little is known about the magnitude and characteristics of MS in people living with HIV (PLWH) in Asian countries in general and China in particular. Using baseline data collected between February 2017 through January 2020 from the Comparative HIV and Aging Research in Taizhou (CHART) cohort in China, we examined MS among 2,227 PLWH and 5,264 matched people without HIV, respectively. MS was defined using the criteria set forth by the International Diabetes Federation (IDF). Approximately 76.7% of PLWH had body mass index (BMI) < 24.0 kg/m^2, significantly higher than people without HIV (50.3%). Among participants with BMI < 24.0 kg/m^2, PLWH had a significantly higher prevalence of MS than people without HIV (20.6% *vs.* 14.5%; aOR: 1.41, 95% CI: 1.19-1.68) overall, and at an age of 18-29 (10.4% *vs.* 3.4%, aOR: 3.49, 95% CI: 1.99-6.11) and 30-44 years (17.3% *vs.* 8.5%, aOR: 2.03, 95% CI: 1.47-2.81), respectively. Among participants with BMI ≥ 24.0 kg/m^2, MS prevalence was not significantly different between PLWH and people without HIV overall, but significantly lower in PLWH than people without HIV for those aged over 60 years (65.9% *vs.* 77.8%, aOR: 0.53, 95% CI: 0.32-0.88). Among PLWH, MS was significantly associated with older age and higher CD4 cell count, and with stavudine (d4T) use only in the group of BMI < 24.0 kg/m^2. Our finding is indicative of a relatively higher risk for early onset of MS among HIV-infected young adults with lower BMI. Research is needed to elucidate the pathogenic mechanism for MS among PLWH.

Keywords metabolic syndrome, body mass index, young adults, HIV infection, China

1. Introduction

Benefited tremendously from antiretroviral therapy (ART) with long life (*1*), people living with HIV (PLWH) increasingly face non-HIV health problems such as metabolic complications including lipodystrophy, dyslipidemia, and insulin resistance (*2*). This cluster of metabolic conditions, combined with elevated blood pressure, is known as metabolic syndrome (MS). Individuals with MS are thought to be more susceptible to cardiovascular disease (CVD) and diabetes mellitus (DM), as well as stroke and cancers (*3-5*).

Many studies have suggested that MS is largely an inflammatory disease. Both adipose tissue (AT) and chemokines or cytokines derived from monocytes contribute to the MS *via* inflammatory pathways (*6*). Among the general population, excessive fat tissue is the main cause of MS development (*7*), and metabolic health often declines as body mass index (BMI) increases. However, the development of MS in PLWH is probably more complex with the coexistence of HIV infection and obesity. Research in developed countries shows that HIV-infected individuals had a high prevalence of being overweight or obesity prior to ART initiation, and 20% progressed to a higher unhealthy weight after ART initiation (*8*). Weight gain after receiving ART, along with HIV infection may accelerate metabolic dysfunction because excessive adipose accumulation and HIV

infection are chronic inflammatory states (*9,10*). Of note, overweight and obesity are not common in all PLWH especially in some Asian countries, where PLWH usually have a relatively low weight compared to the general population ever after ART initiation (*11*). For PLWH with low BMI, the effect of HIV-infection on development of MS is probably not overshadowed by that of excessive adipose tissue.

The global literature has revealed diverse estimates of prevalence of MS among PLWH (*12*). However, there is no definitive conclusion about the association between HIV infection and MS (*13-16*). Meanwhile, data on prevalence and risk factors for MS among PLWH with relatively lower or normal weight are very limited. Therefore, we conducted a cross-sectional study to: *i*) estimate the prevalence of MS and its association with different categories of BMI; *ii*) examine correlates of MS among PLWH in mainland China.

2. Materials and Methods

2.1. Study design and participants

Using baseline data collected between February 2017 through January 2020 from an ongoing cohort known as the "Comparative HIV and Aging Research in Taizhou" (CHART) in Taizhou prefecture, Zhejiang province, east China, we examined MS among 2,227 PLWH and 5,264 individuals without HIV, respectively. The CHART is an ongoing prospective cohort study of HIV and age-related co-morbidities among PLWH and people without HIV; details of baseline characteristics of CHART have been described previously (*17,18*).

According to China treatment guidelines, the first-line of HIV treatment regimen for ART-naïve patients consists of two nucleotide reverse-transcriptase inhibitors (NRTIs) including lamivudine (3TC), along with zidovudine (AZT) or stavudine (d4T, replaced with tenofovir disoproxil fumarate [TDF] since 2010), and one non-nucleotide reverse-transcriptase inhibitor (NNRTIs) namely nevirapine (NVP) or efavirenz (EFV). Patients who experience side effects or drug resistance from NNRTIs may receive the two NRTIs mentioned above plus lopinavir (LPV) – a proteinase inhibitor (PI).

The study was approved by the Institutional Review Board of Fudan University School of Public Health, Shanghai, China. All participants have subscribed to the informed consent.

2.2. Data collection

Basic demographic and lifestyle characteristics of all participants were collected using a structured questionnaire, including sex, age, physical activity, smoking status, and alcohol use. Age groups were categorized by cut-offs of 18-29, 30-44, 45-59, and 60 and above according to the previous paper (*17*). Smoking status was classified as "never", "previous" or "current", with current smoking defined as having smoked at least one cigarette in the past 30 days. Regular exercise was defined as having engaged in exercise more than three times per week. Regular alcohol use was defined as often or always drinking alcohol in the past month. HIV-related information (*i.e.*, date of HIV diagnosis, CD4 count, date of ART initiation, and ART regimen) was extracted from the national "Comprehensive Response Information Management System (CRIMS)" for HIV patients in China.

Height, weight, waist circumference, and blood pressure were measured by trained public health workers. Measurement of blood pressure was performed two times 5 minutes apart and the mean value was recorded. BMI calculated as body weight divided by height squared (kg/m^2) was categorized into underweight/normal weight (< 24.0 kg/m^2), or overweight/obese (≥ 24.0 kg/m^2) according to the criteria set forth by the "Working Group on Obesity in China" (*19*). Venous blood samples were collected for glycated hemoglobin (HbA1c) and lipids measurement.

2.3. Definition of metabolic syndrome (MS)

According to the "International Diabetes Federation" (IDF) (*20*), MS is defined as the presence of at least three of the following five components: *i*) an increased waist circumference (Asian males > 90 cm; Asian females > 80 cm); *ii*) blood pressure ≥ 130/85 mmHg or hypertension based on self-report and/or local hospital records; *iii*) raised triglycerides (TG) levels ≥ 150 mg/dL (1.7 mmol/L); *iv*) reduced high density lipoprotein (HDL) < 40 mg/dL (1.03 mmol/L) in males or HDL < 50 mg/dL (1.29 mmol/L) in females; and *v*) elevated level of HbA1c > 5.7 % (*21*) or diabetes based on self-report and/or local hospital records.

2.4. Statistical analysis

All analyses were carried out using Stata 15.0 (Stata Corp., College Station, TX, USA). Descriptive data were expressed as mean with standard deviation, median (IQR) or tabulated as the number and percentage. Differences between groups were assessed by χ^2 test for categorical variables and by *t*-test or Kruskal-Wallis test for continuous variables, where appropriate. Logistic regressions were performed to evaluate the association of HIV-infection and MS within different BMI (< 24.0 or ≥ 24.0 kg/m^2) and age groups. HIV-specific correlates (*e.g.*, CD4) of MS were also examined among PLWH. Variables with $p < 0.200$ in univariate analysis and *a priori* were included in final multivariate analysis.

3. Results and Discussion

A total of 7,491 participants aged 18 to 75 years were

included in this analysis. They were mostly male (73.7%) with a mean age of 44.2 (± 14.3) years. Table 1 shows the demographic, lifestyle, and clinical characteristics of the PLWH (n = 2,227) and their negative counterparts (n = 5,264) stratified by BMI category (< 24.0 or ≥ 24.0 kg/m^2). Compared to people without HIV, PLWH were younger with lower BMI, less likely to be smokers and alcohol users. 76.7% of PLWH had BMI < 24.0 kg/m^2, significantly higher than people without HIV (50.3%). These two groups were significantly different in blood pressure and biochemical characteristics (Table 1). PLWH had a median (IQR) value of current CD4 count of 425 (277-577) count/μL. The median (IQR) duration of HIV infection and ART usage prior to recruitment into the study was 2.3 (0.6-5.1) years and 1.7 (0.4-4.0) years, respectively. Protease inhibitors were used by 28.7% of PLWH, whereas the rest of the sample (71.3%) used NNRTI-based regimens. For past or current use of the NRTI, 6.8% of those with BMI < 24.0 kg/m^2 had used stavudine (d4T), significantly higher than 4.4% among those with BMI ≥ 24.0 kg/m^2 (Table 1).

The prevalence of MS was 35.0% (95% CI: 33.9-36.1) for the entire sample. The global literature is equivocal concerning whether the prevalence of MS is higher among PLWH than in the general population (*15,16,22*). In our study, the overall MS prevalence is lower among PLWH (29.0%, 95% CI: 27.1-30.9) than people without HIV (37.5%, 95% CI: 36.2-38.9), partially due to lower weight and BMI of HIV cases. Nevertheless, a significantly higher risk for MS was observed among PWLH with BMI < 24.0 kg/m^2 than their HIV-negative counterparts (20.6% *vs.* 14.5%; aOR: 1.41, 95% CI: 1.19-1.68, p < 0.001) (Table 2), a finding similar to previous research (*23*).

MS is an age-related morbidity where incidence tends to go up with increased age (*24*). The prevalence of MS increased with age for both PLWH and people without HIV, from 16.0 % at 18-29 years to 39.2% at 60-72 years among PLWH, and from 16.7% to 60.1% correspondingly among people without HIV. Similar patterns were observed among participants within both BMI strata, *i.e.*, BMI < 24.0 kg/m^2 or BMI ≥ 24.0 kg/m^2 (Table 2). Given the effect of BMI (as a moderator) on HIV infection with MS, we statistically contrasted the BMI- and age-specific prevalence of MS among participants with BMI < 24.0 kg/m^2 and BMI ≥ 24.0 kg/m^2, respectively (Figure 1 and Table 2). A higher prevalence of MS was observed among PLWH than

Table 1. Characteristics of PLWH and people without HIV stratified by BMI category

Variables	Total		BMI < 24.0 kg/m^2		p	BMI ≥ 24.0 kg/m^2		p
	HIV+ (n = 2,227)	HIV- (n = 5,264)	HIV+ (n = 1,707)	HIV- (n = 2,645)		HIV+ (n = 520)	HIV- (n = 2,619)	
Male	1,737 (78.0)	3,782 (71.9)	1,332 (78.0)	1,751 (66.2)	< 0.001	405 (77.9)	2031 (77.6)	0.867
Age, yrs	44.1 ± 14.0	44.2 ± 14.4	43.7±14.3	41.8 ± 14.6	< 0.001	45.6 ± 13.1	46.6 ± 13.7	0.090
18-29	438 (19.7)	1,001 (19.0)	367 (21.5)	651 (24.6)	0.003	71 (13.7)	350 (13.4)	0.139
30-44	767 (34.4)	1,808 (34.4)	571 (33.5)	948 (35.8)		196 (37.8)	860 (32.8)	
45-59	652 (29.3)	1,567 (29.8)	487 (28.5)	672 (25.4)		165 (31.7)	895 (34.2)	
60-75	370 (16.6)	888 (16.9)	282 (16.5)	374 (14.1)		88 (16.9)	514 (19.6)	
Smoking status					< 0.001			< 0.001
Never	1,349 (60.6)	2,979 (56.6)	1,025 (60.1)	1,608 (60.8)		324 (62.3)	1371 (52.4)	
Previous	269 (12.1)	498 (9.5)	198 (11.6)	193 (7.3)		71 (13.7)	305 (11.7)	
Current	609 (27.4)	1,787 (34.0)	484 (28.3)	844 (31.9)		125 (24.0)	943 (36.0)	
Regular exercise	801 (36.0)	1,650 (31.3)	624 (36.6)	812 (30.7)	< 0.001	177 (34.0)	838 (32.0)	0.363
Regular alcohol use	207 (9.3)	1,043 (19.8)	159 (9.3)	451 (17.1)	< 0.001	48 (9.2)	592 (22.6)	< 0.001
SBP, mmHg	124.4 ± 15.4	126.8 ± 17.8	123.0 ± 14.6	121.0 ± 16.2	< 0.001	130.0 ± 16.6	132.6 ± 17.4	0.003
DBP, mmHg	76.8 ± 10.2	78.1 ± 17.5	75.8 ± 9.8	74.8 ± 16.7	< 0.001	80.2 ± 10.8	81.4 ± 17.7	0.072
TG, mmol/L	2.16 ± 1.80	2.23 ± 1.76	1.95 ± 1.40	1.71 ± 1.14	< 0.001	2.87 ± 2.60	2.76 ± 2.09	0.375
HDL, mmol/L	1.10 ± 0.34	1.18 ± 0.30	1.12 ± 0.36	1.26 ± 0.32	< 0.001	1.03 ± 0.29	1.09 ± 0.25	< 0.001
HbA1c, %	5.28 ± 0.80	5.71 ± 0.86	5.25 ± 0.77	5.54 ± 0.67	< 0.001	5.37 ± 0.87	5.88 ± 0.99	< 0.001
WC, cm	82.1 ± 7.83	82.5 ± 10.7	79.8 ± 6.6	75.3 ± 7.4	< 0.001	89.6 ± 6.8	89.8 ± 8.3	0.867
BMI, kg/m^2	22.0 ± 3.06	24.2 ± 3.7	20.8 ± 1.9	21.2 ± 1.9	< 0.001	26.1 ± 2.5	27.1 ± 2.6	< 0.001
Metabolic syndrome	646 (29.0)	1,976 (37.5)	351 (20.6)	383 (14.5)	< 0.001	295 (56.7)	1,593 (60.8)	0.082
Current CD4, count/μL[†‡]	425 (277-577)	-	420 (276-571)	-		434 (279-597)	-	0.045
Years since HIV diagnosis[†]	2.3 (0.6-5.1)	-	2.3 (0.6-5.3)	-		2.2 (0.6-4.8)	-	0.408
Duration on ART, yrs[†]	1.7 (0.4-4.0)	-	1.7 (0.4-4.2)	-		1.7 (0.5-3.6)	-	0.192
< 1	913 (41.0)	-	701 (41.1)	-		212 (40.8)	-	0.138
1~ < 3	567 (25.5)	-	419 (24.6)	-		148 (28.5)	-	
≥ 3	747 (33.5)	-	587 (34.4)	-		160 (30.8)	-	
Ever using d4T	139 (6.2)	-	116 (6.8)	-		23 (4.4)	-	0.050
Ever using PIs	640 (28.7)	-	485 (28.4)	-		155 (29.8)	-	0.538

ART, antiretroviral therapy; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; HDL, high density lipoprotein; HbA1c, glycated hemoglobin; WC, waist circumference; BMI, body mass index; d4T, stavudine; PIs, Protease Inhibitors. p-value for chi-square test or Kruskal-Wallis test, where it is appropriate. [†]Median (interquartile range); [‡]16 observations were missing.

Table 2. Association of HIV Infection with Metabolic Syndrome by BMI Category and Age groups

Age Groups[†]	BMI < 24.0 kg/m^2			BMI ≥ 24.0 kg/m^2		
	MS Prevalence (%)	aOR (95% CI)	*p*	MS Prevalence (%)	aOR (95% CI)	*p*
18-29	5.9 (60/1,018)	3.49 (1.99-6.11)	< 0.001	42.0 (177/421)	1.25 (0.74-2.10)	0.405
30-44	11.9 (180/1,519)	2.03 (1.47-2.81)	< 0.001	53.8 (568/1,056)	1.01 (0.73-1.38)	0.964
45-59	23.6 (273/1,159)	1.25 (0.94-1.66)	0.122	64.6 (685/1,060)	0.78 (0.55-1.10)	0.155
60-75	33.7 (221/656)	0.80 (0.57-1.13)	0.207	76.1 (458/602)	0.53 (0.32-0.88)	0.014
Total[‡]	16.9 (734/4,352)	1.41 (1.19-1.68)	< 0.001	60.1 (1888/3,139)	0.88 (0.72-1.07)	0.189

BMI, Body Mass Index; MS, metabolic syndrome ; aOR, adjusted Odds Ratio. [†]Adjusted for gender, smoking status, exercise and alcohol use for each age-specific subgroup. [‡]Adjusted for age, gender, smoking status, exercise and alcohol use for the total sample.

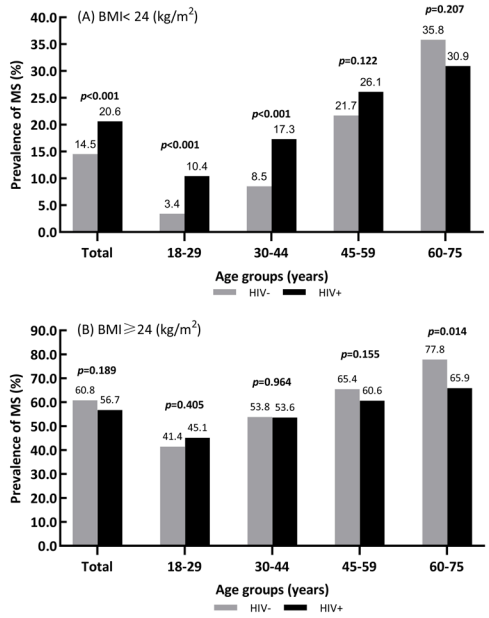

Figure 1. Prevalence of metabolic syndrome across age groups in PLWH and people without HIV. (A) the comparison among individuals with BMI < 24.0 kg/m^2; **(B)** the comparison among individuals with BMI ≥ 24.0 kg/m^2. The *p*-values were obtained from multivariable logistic regression models adjusted for age, gender, smoking status, alcohol use and exercise.

people without HIV within the age group of 18-29 years (10.4% *vs.* 3.4%, aOR: 3.49, 95% CI: 1.99-6.11); 30-44 years (17.3% *vs.* 8.5%, aOR: 2.03, 95% CI: 1.47-2.81); and 45-59 years (26.1% *vs.*21.7%, aOR: 1.25, 95% CI: 0.94-1.66). The positive association of HIV infection with MS is evident at younger ages, possibly because the influence of aging itself surrenders to the impact of HIV infection on MS at a younger age. Besides aging itself, the effect of HIV infection might also be overshadowed by the effect of prevalent traditional risk factors on MS, which therefore render the insignificant association of HIV infection and MS in older age groups.

However, no significant difference in the presence of MS was observed among PLWH and their negative counterparts with BMI ≥ 24.0 kg/m^2 within age groups 18-29 and 30-44, which might correlate with the role of fat tissue in the development of MS. Excessive fat is a major cause of MS for both the general population and PLWH. However, adipose tissue not only acts as a storage and secretion organ involved in energy regulation and metabolism, but also functions as an immune regulator involved in many biological processes (*25,26*). Accordingly, the harmful nature of the HIV virus itself and/or use of ART may somehow be covered by the impact of excessive adipose tissue (*27*), which might lead to a similar prevalence of MS in PLWH comparable to the general population among the overweight or obese group. Nonetheless, we observed a lower prevalence of MS among overweight or obese PLWH within the 60-75 age group. As the development of MS in PLWH is the result of the interplay between traditional risk factors, viral load, and type of ART usage (*28*), it is possible that diverse factors are involved in this complex biological process that then lead to this phenomenon. The reasons remain to be investigated.

In PLWH (Table 3), factors significantly associated with MS in individuals with BMI < 24.0 kg/m^2 included all older age groups, current CD4 ≥ 500 count/μL and ever using d4T. In individuals with BMI ≥ 24.0 kg/m^2, a significantly increased risk was only observed in 45-59 and 60-75 age groups, as well as in current CD4 ≥ 500 count/μL. The overall findings on MS among PLWH are consistent with those often observed in the general population where MS risks increase with age (*24*), although no sex difference was observed in our study.

Similar to previous studies (*29*), no significant association of duration of HIV infection and duration on ART with MS was observed in this study. Considering some ART regimens have a hyperlipidemic effect, the combination of antiretroviral drugs (ARVs) rather than the duration on ART and HIV infection may exert more effects on MS. Given that stavudine (d4T) could induce lipodystrophy and central fat gain (*30*), it is not surprising that ever using d4T is associated with MS. It is noted that such association was only significant among the underweight or normal weight PLWH after adjustment for other covariates, which suggests the potential impact of fat accumulation itself on MS among overweight or obese patients. While the use of PIs for treatment might increase risks for MS (*13*), higher prevalence of MS was only observed among underweight or normal weight

Table 3. Logistic Regression Analysis of Factors Associated with Metabolic Syndrome among PLWH

Variables	BMI < 24.0 kg/m^2				BMI ≥ 24.0 kg/m^2			
	cOR (95%CI)	*p*	aOR (95% CI)	*p*	cOR (95% CI)	*p*	aOR (95% CI)	*p*
Male	1.13 (0.85-1.49)	0.394	0.97 (0.71-1.32)	0.834	1.37 (0.89-2.09)	0.150	1.33 (0.83-2.12)	0.232
Age, yrs								
18-29	1		1		1		1	
30-44	1.82 (1.22-2.71)	0.003	1.80 (1.19-2.71)	0.005	1.41 (0.82-2.43)	0.220	1.43 (0.81-2.51)	0.214
45-59	3.05 (2.06-4.52)	< 0.001	3.10 (2.06-4.64)	< 0.001	1.88 (1.07-3.29)	0.028	1.97 (1.09-3.54)	0.024
60-75	3.86 (2.54-5.88)	< 0.001	4.07 (2.63-6.31)	< 0.001	2.36 (1.24-4.48)	0.009	2.41 (1.24-4.67)	0.009
Smoking status								
Never	1		1		1		1	
Previous	1.51 (1.07-2.14)	0.021	1.20 (0.82-1.76)	0.353	1.49 (0.87-2.54)	0.143	1.60 (0.91-2.81)	0.106
Current	0.96 (0.73-1.26)	0.754	0.86 (0.63-1.16)	0.314	1.03 (0.68-1.56)	0.886	1.13 (0.72-1.77)	0.590
Regular exercise	1.00 (0.78-1.27)	0.969	-		1.09 (0.76-1.58)	0.629	-	
Regular alcohol use	0.93 (0.62-1.40)	0.727	-		1.18 (0.64-2.17)	0.589	-	
Current CD4, count/μL								
< 200	1		1		1		1	
200-500	0.85 (0.59-1.22)	0.376	0.93 (0.64-1.35)	0.705	1.29 (0.75-2.21)	0.361	1.50 (0.85-2.63)	0.162
≥ 500	1.20 (0.84-1.73)	0.311	1.49 (1.02-2.19)	0.041	1.65 (0.97-2.83)	0.067	2.03 (1.15-3.60)	0.015
Years since HIV diagnosis								
< 1	1		-		1			
1~< 3	0.74 (0.53-1.05)	0.093	-		0.95 (0.61-1.50)	0.831	-	
≥ 3	1.16 (0.89-1.51)	0.262	-		1.04 (0.69-1.55)	0.861	-	
Duration on ART, yrs†								
< 1	1		1		1		1	
1 ~ < 3	0.91 (0.67-1.24)	0.551	0.99 (0.71-1.38)	0.947	0.81 (0.53-1.24)	0.336	0.81 (0.51-1.27)	0.349
≥ 3	1.21 (0.92-1.58)	0.167	0.97 (0.71-1.33)	0.868	0.96 (0.63-1.46)	0.848	1.01 (0.63-1.61)	0.979
Ever using d4T	2.45 (1.65-3.65)	< 0.001	2.31 (1.47-3.62)	< 0.001	0.69 (0.30-1.59)	0.380	0.60 (0.24-1.48)	0.269
Ever using PIs	1.08 (0.83-1.39)	0.571	1.19 (0.90-1.58)	0.228	1.21 (0.83-1.77)	0.327	1.25 (0.82-1.90)	0.298

Variables with $p < 0.200$ in univariate analysis were included in multivariate regression. All models were adjusted for gender and age. cOR, crude Odds Ratio; aOR, adjusted Odds Ratio; BMI, Body Mass Index; ART, Antiretroviral Therapy; d4T, Stavudine; PIs, Protease Inhibitors. †The variable 'years since diagnosis' was not included in multivariate regression for collinearity with duration on ART.

patients.

Using baseline data from an ongoing, large-scale cohort of PLWH and people without HIV, we provide some of the first empirical evidence on MS and HIV in mainland China. Our findings not only reinforce the role of HIV infection in the pathogenesis of MS, but also suggest the effect of modifications of BMI and age involved in MS in a study population.

Our study had some limitations. Due to the cross-sectional nature of this study, we cannot determine the temporal relationship between HIV infection and MS. Body composition measured by computed tomography (CT) or magnetic resonance imaging (MRI) may be a more appropriate indicator for metabolic status, which was not available in this study. However, CT and MRI are not applicable in source-limited areas compared with simple BMI. Also, information on potential confounding factors that may influence MS such as dietary habits and meditation use were not available during the study period. Nonetheless, our data are likely to be generalizable or heuristic as is from a large-scale cohort with PLWH and people without HIV within a diverse age range.

In conclusion, this study provides evidence that HIV infection is significantly associated with MS, especially among normal or underweight young Chinese. Our finding is indicative of the risk of early onset of MS among HIV-infected young adults with lower BMI. Research is warranted to elucidate the pathogenic mechanisms for MS among PLWH.

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[§]These authors contributed equally to this work.

*Address correspondence to:
Na He, Department of Epidemiology, School of Public Health, Fudan University, P.O.Box 289, 138 Yi Xue Yuan Road, Shanghai 200032, China.
E-mail: nhe@fudan.edu.cn

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Letter

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Perception of mutual aid and its related factors: a study of Japanese high school students

Tomoko Yokomizo[1], Kumi Kanno[2], Akemi Yamagishi[3], Toshi Nagata[4,*]

[1] Social Welfare Corporation Tenryu Kohseikai, Hamamatsu, Japan;
[2] Department of Clinical Nursing, School of Nursing, Fukushima Medical University, Fukushima, Japan;
[3] Department of Public Health, Keio University School of Medicine, Tokyo, Japan;
[4] Department of Health Science, Faculty of Nursing, Hamamatsu University School of Medicine, Hamamatsu, Japan.

SUMMARY Japan is a super-ageing country. Constructing the community-based integrated care system in local communities is urgently needed. Mutual aid in local communities is critical for this system. In order to clarify the status of perception of mutual aid in Japanese high school students and to clarify the factors related to the formation of the perception, we conducted a questionnaire study of high school students in a city in Japan (n = 8,687). The results indicate that Japanese high school students show a tendency to have perception of mutual aid for local people (70.8%) rather than the local area (38.9%). Significantly fewer male students have perception of mutual aid than female students ($p < 0.01$). Factors that affected the perception significantly ($p < 0.05$) were: *i*) willingness to stay in the local area for 10 more years, *ii*) recognition of persons in need of care in the local area, *iii*) memories of experiencing communication with handicapped and/or elderly people, and *iv*) experience of taking care of local children. It is important to create opportunities for high school students to communicate with local residents, especially handicapped and/or elderly people in order to foster students' perception of mutual aid.

Keywords community-based integrated care system, place attachment, local people, local area

Japan has been categorized as a super-aged society. The percentage of the population over 65 years of age in Japan is predicted to be 40.5% by 2055 (*1*). A community-based integrated care system is defined as a system that includes not only medical and nursing care but also welfare services in order to guarantee safety, security and health according to needs in a local area (*2,3*).

It is indispensable to create the system as a mechanism to provide these services comprehensively and continuously in local communities. The system is based on four different care concepts: governmental care (Ko-jo), social solidarity care (Kyo-jo), self-help (Ji-jo), and mutual aid (Go-jo) (*4*). Mutual aid (Go-jo) is the voluntary mutual support of local people. As it is difficult to expect to greatly expand social solidarity care, great expectation is given to mutual aid conducted by local residents and others (*5*). We examined the actual status of Japanese high school students' perception of mutual aid and the related factors. The reason that we focused on high school students is that they constitute an essential generation that will be responsible for a community-based integrated care system in the future.

We surveyed high school students in Hamamatsu city in Japan (the population as of Feb 1, 2020 was 802,201). The survey was conducted by an anonymous self-administered questionnaire. We sent a research request document to the principals of all 26 high schools in Hamamatsu and received consent from 19 high schools. Then, we sent the questionnaires and performed the survey, and the answered questionnaires were returned by mail. The survey was conducted between January and March of 2016.

The questionnaire consists of the following items: 1) degree of perception of mutual aid, 2) demographic factors, 3) activity experiences from elementary school through high school, 4) recognition of people in need of care in the local area, 5) attachment to the local area (willingness to continue to live in the local area), 6) sympathy for handicapped people (a "multi-dimensional attitude measure for people with disabilities" created by Kusunoki (*6*) was used), 7) sympathy for elderly people (a Japanese shortened version (*7*) of the Frabonie Ageism Scale was used), and 8) empathy with other people (a multi-dimensional empathy scale created by Tobari (*8*) was used). SPSS ver. 21.0 (Japan IBM, Tokyo,

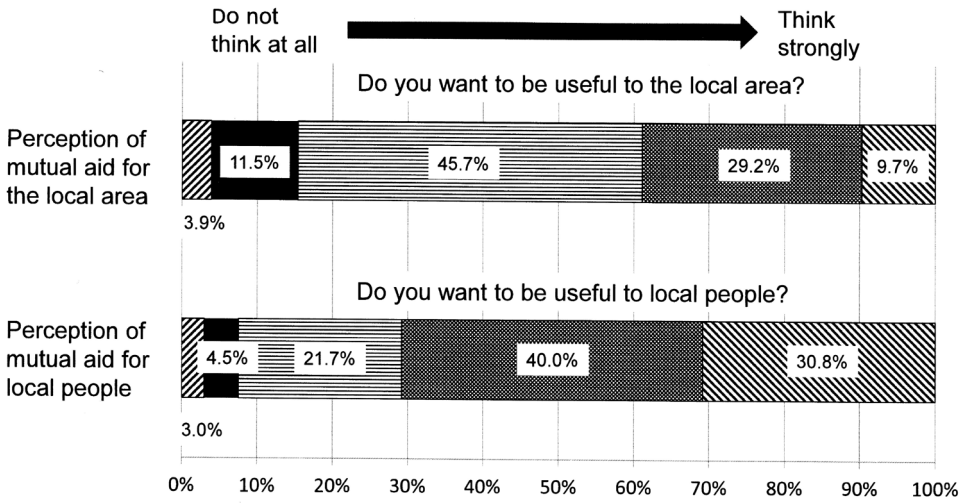

Figure 1. Degree of perception of mutual aid in Japanese high school students. The percentages of the degree of perception of mutual aid for the local area and local people in Japanese high school students are shown. Students were asked the questions: "Do you want to be useful to the local area?" and "Do you want to be useful to local people?" and they were answered based on the five-point scale: 1. "I do not think so at all", 2. "I do not think so", 3. "I am not sure whether I think so or not", 4. "I think so", or 5. "I strongly think so".

Japan) was used for statistical analyses. This study was conducted with the approval of the ethics committee of Hamamatsu University School of Medicine (approval number: E15-245).

The number of questionnaires distributed was 9,023. The questionnaire collection amount was 8,687 (a recovery rate of 96.3%). Among them, 7,136 responses in which all items were answered were used for the analyses. The percentage of students who have perception of mutual aid towards local people (70.8%) is higher than that towards the local area (38.9%) (Figure 1). Hidalgo and Hernandez (*9*) reported that social attachment to a house, neighborhood, or city was stronger than physical attachment to them. Students who have lived in their current location for more than 10 years tend to have perception for both the local area and local people more strongly compared with students who have lived for less than 10 years ($p < 0.01$ by chi-squared test). Living longer in the local area will strengthen the "place attachment" of students. The proportion of male students who have perception of mutual aid was significantly lower than that of female students ($p < 0.01$ by chi-squared test).

A multiple logistic regression analysis was performed with "perception of mutual aid to both the local area and local people" as a subordinate variable and the possible influence factors as independent variables. The factors that affected perception of mutual aid significantly ($p < 0.05$) were: *i*) willingness to stay in the local area for 10 more years, *ii*) recognition of persons in need of care in the local area, *iii*) memories of experiencing communication with handicapped and/or elderly people, and *iv*) experience of taking care of local children. It is important to create opportunities for high school students to communicate with local residents, especially handicapped and/or elderly people in order to foster students' perception of mutual aid. Young people's volunteer activity may be effective for the purpose. Regional welfare facilities may play a role in fostering students' perception of mutual aid by implementing local events.

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**Address correspondence to:*
Toshi Nagata, Department of Health Science, Faculty of Nursing, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192, Japan.
E-mail: tnagata@hama-med.ac.jp

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Letter

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Half depletion of Foxp3+ regulatory T cells by diphtheria toxin for long-term study *in vivo*

Xuemin Qiu[1,2,3,§], Wing Ting Leung[1,2,3,§], Hans-Jürgen Gober[4], Lisha Li[1,2,3], Na Zhang[1,2,3], Nan Chu[1,2,3], Ling Wang[1,2,3,*]

[1] Laboratory for Reproductive Immunology, Hospital & Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai, China;
[2] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[3] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China;
[4] Department of Pharmacy, Neuromed Campus, Kepler University Hospital, Linz, Austria (Present address: Pharmaceutical Outcomes Programme, British Columbia Children's Hospital, 938 West 28th Avenue, Vancouver BC, Canada).

SUMMARY Depletion of regulatory T cells (Tregs) is an appropriate approach to study the function of Tregs *in vivo*, and most previous studies have focused on complete depletion. The purpose of the current study was to determine an appropriate dose and timing for half depletion of Tregs *in vivo*. DETREG (DEpletion of REGulatory T cells) mice were produced and injected with different doses of diphtheria toxin (DT) for 7 days and 14 days. The mice were then sacrificed to collect the spleen and mesenteric lymph nodes (MLN) for analysis using flow cytometry. Foxp3+eGFP+ cells were significantly reduced by DT injection. A dose of 5 ug/kg DT led to half depletion and no deaths. A DT dose of 25, 50, or 100 ug/kg led to a progressively higher depletion rate but also a higher mortality rate. In conclusion, a low dose of DT is effective for half depletion of Tregs and long-term study. Half depletion of Tregs may become a new method for the future study of Tregs *in vivo*.

Keywords half depletion of Foxp3+ regulatory T cells, DETREG mice, Diphtheria toxin (DT)

Researchers have focused on the modulation of regulatory T cells (Tregs) for years because of the important role of Tregs *in vivo* (*1*). However, specific *in vivo* targeting of Tregs is precluded because of the lack of appropriate markers. Now that Foxp3 has been discovered, the development of mouse models of Treg-specific depletion is feasible (*2*). DETREG (DEpletion of REGulatory T cells) mice carry a diphtheria toxin receptor - enhanced green fluorescent protein (DTR-eGFP) transgene under the control of an additional Foxp3 promoter, thereby allowing specific depletion of Tregs by diphtheria toxin (DT) at any desired point of time during any ongoing immune responses (*3*). Most previous studies have focused on complete depletion of Tregs, which facilitates the study of Tregs *in vivo*. However, half depletion of Tregs may be a new method for studying the function of Tregs *in vivo*.

The aim of the current study was to explore how to use DT to half deplete Tregs *in vivo* and to determine a proper dose and timing in order to facilitate the long-term study of Tregs.

DT was purchased from Sigma-Aldrich. Flow cytometry antibodies including fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-Foxp3-PE, anti-CD25-APC, and their corresponding isotype controls were purchased from eBioscience (San Diego, CA, USA). RT-PCR reagents were purchased from Applied Biosystems (Darmstadt, Germany).

To create a DETREG (Foxp3DTR-EGFP) mouse model, two transgene mouse models were used, pCAG-STOP-DTR-2A-EGFP mice (denoted as DTR-EGFP mice, product number: BCG-TO-0001) were purchased from Beijing Biocytogen Co., Ltd (Beijing, China), and B6.129(cg)-Foxp3$^{tm4(YFP/cre)Ayr}$/J mice (denoted as Foxp3-Ycre mice) were obtained from the Chinese Academy of Sciences (Dr. Qi-bin Leng's Lab, Shanghai, China). All mice were bred and reproduced in an animal facility of the Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University under specific pathogen-free conditions. This study was approved by the Animal Care and Ethics Committee of the Department of Laboratory Science, Fudan University (approval no. 2020 Obstetrics & Gynecology Hospital JS020). DETREG mice genotyping Primers (5'-3'): Rosa-GT-F: AGTCGCTCTGAGTTGTTATCAG, Rosa-GT-R: TGAGCATGTCTTTAATCTACCTCGATG; WPRE-F: GCATCGATACCGTCGACCTC, WPRE-R: GCTGTCCATCTGCACGAGAC; Ycre-WT-F:

CCTAGCCCCTAGTTCCAACC, Ycre-WT-R: AAGGTTCCAGTG CTGTTGCT; Ycre-MU-F: AGGATGTGAGGGACTACCTCCTGTA, Ycre-MU-R: TCCTTCACTCTGATTCTGGCAATTT.

After mice were sacrificed, spleen and mesenteric lymph node (MLN) cells were analyzed using a flow cytometer (Becton Dickinson, Palo Alto, CA, USA).

Continuous variables were expressed as the mean ± SEM. Continuous variables were analyzed using a Student's *t*-test for two groups and a one-way ANOVA for multiple groups. All analyses were performed using the SPSS 19.0 Statistical Package for the Social Sciences. $P < 0.05$ was considered statistically significant.

Genotyping of DETREG mice with RT-PCR is shown in Figure 1. The GFP+ cell and Foxp3+ cell populations in the spleen and MLNs were analyzed using FCM. The number of GFP+ Tregs was much higher in DETREG mice compared to that in wild-type (WT) mice (13.3% *vs*. 3.01% in the spleen, 16.1% *vs*. 0.87% in MLNs, $p < 0.05$). However, the number of Foxp3+ cells did not differ, suggesting that a DETREG mouse model was successfully created.

In order to achieve half depletion, DETREG and WT mice were injected i.p. with DT (5, 25, 50, or 100 ug/kg) every 48 h. After mice were sacrificed, Foxp3+ and GFP+ cells in the spleen and MLNs were analyzed. As shown in Figure 2, both GFP+ cells in the spleen and MLNs decreased after DT injection. There was a severe drop in Foxp3+ cell as the dose increased, suggesting a higher DT dose led to a higher depletion rate. However, a high dose led to severe weight loss and a high mortality rate in the 50 ug/kg and 100 ug/kg groups (data not shown).

On the day of the eighth DT injection (day 14), the number of Foxp3+ cells did not differ in the DT and WT groups, suggesting a rebound of Tregs in DETREG mice (data not shown).

DT injection allows for a highly specific depletion of Tregs at any desired time point during an ongoing immune response. Most researchers use a large dose of DT to completely deplete Tregs in order to study their function *in vivo* (*4*). However, complete depletion of Tregs *in vivo* causes a fatal autoimmune pathology in mice (*5,6*). The aim of the current study was to determine an appropriate dose and timing for half depletion of Tregs *in vivo*, while previous studies only achieved complete depletion for a short period of time.

In conclusion, previous studies of Tregs using DETREG mice focused on complete depletion for a short period of time. In contrast, the current study achieved a half depletion of Tregs for a prolonged period of time. This approach is potentially a new way to study Tregs *in vivo* in the future, offering new possibilities for research on Tregs and especially for long-term study of Tregs.

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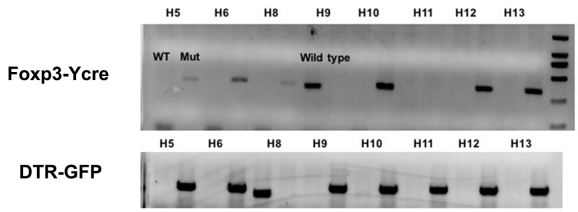

Figure 1. A DEREG mice model has successfully been created and verified using RT-PCR. DETREG mice were genotyped using RT-PCR. For DTR mice: WT=Rosa-GT-F/R: 469 bp; Mutant=WPRE-F/R: 561 bp; For Ycre mice: Mutant = ~346 bp; Heterozygote = 322 bp and ~346 bp; Wild type = 322 bp.

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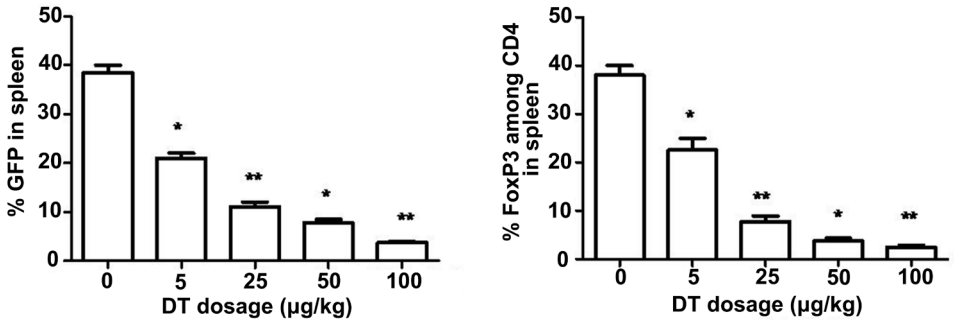

Figure 2. Depletion of Tregs by DT was dose-dependent. DEREG and WT mice were injected i.p. with different doses of DT (0, 5, 25, 50, 100 ug/kg) every 48 h. Mice were sacrificed on the day following the fourth DT injection because of the significant mortality rate in the DT high-dose groups. GFP+ and Foxp3+ cells were counted using FCM. (*$P < 0.05$, **$P < 0.01$)

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Ling Wang, Obstetrics & Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China.
E-mail: Dr.wangling@fudan.edu.cn

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Letter

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Latest updates on COVID-19 vaccines

Qian Li, Hongzhou Lu*

Department of Infectious Diseases, Shanghai Public Health Clinical Center, Shanghai, China.

SUMMARY The ongoing outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has raised a grave concern and a severe global health burden. Since no effective drugs have been approved for satisfactory prevention and treatment, the development of COVID-19 vaccines has attracted global attention. To date, a large number of COVID-19 vaccines are being rapidly developed worldwide, with thirteen candidates in Phase 3 trials, 52 tested in clinical trials, and 162 in preclinical evaluation. Here, we summarize the latest progress of all 13 COVID-19 vaccines in Phase 3 trails. Furthermore, some vaccines have received approval or emergency use approvals. We focus on the potential issues related to vaccination including vaccine acceptance, vaccine promotion, and vaccine distribution.

Keywords COVID-19, SARS-CoV-2, vaccine acceptance, vaccine promotion, vaccine distribution

The pandemic of Coronavirus Disease 2019 (COVID-19) has raised a severe global threat. The causative pathogen novel SARS-CoV-2 (previously called 2019-nCoV) was first identified in Wuhan, China in early December 2019 and has been recently named as the Coronavirus Disease-2019 (COVID-19) by the World Health Organization (*1,2*). As of 17 December 2020, more than 74,087,090 cases of COVID-19 have been confirmed in over 200 countries and 6 continents, resulting in approximately 1,646,687 deaths (*3*). Since no effective vaccine existed, the development of a safe and effective COVID-19 vaccine, urgent for disease control, has attracted global attention.

Most recently, thirteen COVID-19 vaccines are being evaluated in Phase 3 clinical trials (Table1). Pfizer/BioNTech first confirmed that the mRNA vaccine (BNT162b2) is 95% effective against COVID-19 within 28 days after the first dose (*4*). Thereafter, the vaccine was authorized for Emergency Use Authorization (EUA) by the FDA, and approved for emergency use in early December in the UK, Canada, and the US, respectively (*5*). Another COVID-19 mRNA vaccine (mRNA-1273) was demonstrated to be 94.5% effective against COVID-19 in Phase 3 clinical trial (*6*). The Food and Drug Administration (FDA) endorsed mRNA-1273 as safe and efficacious on 15 December 2020 (*7*).

Four Adenovirus vaccines are in Phase 3 clinical trials. They are AZD1222 from AstraZeneca/Oxford, Ad26.COV2.S from Johnson & Johnson/Janssen, Ad5-nCoV from CanSino Biologics, and Gam-COVID-Vac from Gamaleya Research Institute. In their press release, AstraZeneca/Oxford reported a 70% reduction of COVID-19 infection in Phase 3 trial of AZD1222, and plan to apply for Emergency Use Authorization (EUA) with the World Health Organization in the coming week (*8-10*). The Ad5-nCoV received Military Specially-needed Drug Approval for use in the Chinese military on June 25, 2020 (*10,11*).

Of note, three companies chose the typical vaccine platform. They are inactivated vaccines, including BBIBP-CorV from the Beijing Institute of Biological Products/Sinopharm, CoronaVac from the Wuhan Institute of Biological Products/Sinopharm, and BBV152 from Bharat Biotech in India. The two vaccines from China, BBIBP-CorV and CoronaVac, submitted for a marketing application to the State Food and Drug Administration at the end of December, 2020 (*10,11*). Two protein subunit vaccines from Novavax and Anhui Zhifei Longcom Biopharmaceutical are in Phase 3 trails now. The Phase 3 trial of NVX-Cov2373 begins with 10,000 participants in both the UK and the U.S in October. The Phase 3 trial of ChiCTR2000040153 begins with 29,000 participants in July, 2020 (*10,11*).

Indeed, the main problem of vaccine development has changed to vaccine acceptance, promotion, and distribution following development of COVID-19 vaccines. It should be another global public-health challenge after ensuring the vaccines safety, efficacy, and durability. Once approved, an equitable plan for vaccine allocation according to demographic structure and underlying recipient conditions is needed (*11*).

Table 1. The thirteen COVID-19 vaccines are being evaluated in Phase 3 clinical trials

Developer	Platform	Type	Partici-pants for Phase III	Storage demands	Approval	Schedule for vaccina-tion	*Ref*
Pfizer/BioNTech BNT162, Tozinameran	RNA	3 LNP-mRNAs	44,000	–70ºC	UK, CAN, USA	Submitted for regula-tory review (02/11) Approve for EUA in UK, CAN, USA (2/12, 9/12, 11/12) 50 million does (at the end of 2020) 130 million (2021)	(*4,5*)
Moderna mRNA-1273	RNA	LNP-encapsulated mRNA	30,000	2–8ºC	unknown	Submitted for regula-tory review (30/11)	(*6,7*)
AstraZeneca/Oxford AZD1222	Non-Replicating Viral Vec-tor	ChA-dOx1-S	65,000	2–8ºC	unknown		(*8,9*)
Johnson & Johnson Ad26.COV2. S	Non-Replicating Viral Vec-tor	Adenovirus Type 26 vector	60,000	2–8ºC	unknown		(*10*)
Novavax NVX-CoV2373	Protein Subunit	Full length recombi-nant SARS CoV-2 glycopro-tein nano-particle vaccine adjuvanted with Matrix M	45,000	2–8ºC	unknown		(*10*)
Sinovac CoronaVac	Inactivated	Inactivated	26,000	2–8ºC	unknown		(*10*)
Wuhan Institute of Bio-logical Products/Sinopharm	Inactivated	Inactivated	15,000	2–8ºC	unknown		(*10*)
Sinopharm Inactivated virus, BBIBP-CorV	Inactivated	Inactivated	50,000	2–8ºC	unknown		(*10*)
Bharat Biotech BBV152	Inactivated	Whole-Virion Inacti-vated	25,000	2–8ºC	unknown	Military Special-ly-needed Drug Ap-proval for emergency use in the Chinese military (25/06)	(*10*)
CanSino Biologics Ad5-nCoV	Non-Replicating Viral Vec-tor	Adenovirus Type 5 Vector	40,000	2–8ºC	unknown		(*10*)
Gamaleya Research In-stitute Gam-COVID-Vac (Sput-nik V)	Non-Replicating Viral Vec-tor	Adeno-based (rAd26-S+rAd5-S)	40,000	–18ºC	unknown		(*10*)
Anhui Zhifei Longcom Biopharmaceuti-cal/Institute of Microbiology, Chinese Academy of Sciences	Protein Subunit	Adjuvanted recombi-nant protein (RBD-Dimer) ex-pressed in CHO cell	29,000	2–8ºC	unknown		(*10*)
Medicago Inc.	VLP	Plant-derived VLP adjuvanted with AS03	20,000	unknown	unknown		(*10*)

Moreover, a well-prepared logistical distribution model, including storage and delivery, is necessary.

Due to the drastic public health interventions taken by China to control COVID-19, only 19 cases were found on December 18, 2020. Hence, only 12.2% of respondents perceived of COVID-19 as a very high risk, which might be problematic for acceptance of COVID-19 vaccination in China (*12*). However, Wang *et al*. reported a high acceptance of COVID-19 vaccination in China (*12*). About ninety-one percent of the participants intend to receive COVID-19 vaccination, with the majority of them accepting both immunization schedules (routine or emergency immunization) and types (domestic or imported) of vaccines (*11*). Contrarily, in countries where COVID-19 is endemic, such as the United States, the willingness to vaccinate against it has dropped from 71% in April to 53.6% in October. It's reported that the proportion of COVID-19 vaccination hesitant and unwilling participants has increased from 10.5% to 14.4%, and 18.5% to 32% respectively. Undecided/ unwilling attitude toward vaccination is closely related to non-degree, black, aged 65+, high income groups, and those with concerns about potential side effects (*13*).

Promotion strategies according to different vaccination acceptance levels, should be taken for a national COVID-19 vaccine promotion program. Five COVID-19 promotion strategies were described by Kevin G et al. These strategies include making vaccines free, making access to valued settings conditional after vaccination, using public endorsements, providing priority access to those who first sign up, and transforming individual vaccination decisions into a public act (*14*).

Almost all vaccine candidates mentioned above require cool storage with different demands. The distribution chains need cooperation from both government and business for cold storage and global transport. Pfizer's shot needs to be stored at around minus 70ºC, Gam-COVID-Vac (Sputnik V) demands a minus 18ºC storage temperature, and the others need to be stored at 2-8ºC. Despite the rapid development and production of vaccines, the distribution of vaccines is still an immense task, especially among socioeconomically deprived groups, rural populations, and un-developed countries. The distribution of vaccines between these regions requires the cooperation and cross-talk of multiple governments and political circles. These regions should be assisted with efficient logistics services.

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*Address correspondence to:
Hongzhou Lu, Department of Infectious Diseases, Shanghai Public Health Clinical Center, 2901 Caolang Road, Shanghai 201508, China.
E-mail: luhongzhou@fudan.edu.cn

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Letter

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Tetracycline plus macrolide: A potential therapeutic regimen for COVID-19?

Masashi Ohe[1,*], Ken Furuya[1], Houman Goudarzi[2]

[1] Department of Internal Medicine, JCHO Hokkaido Hospital, Sapporo, Japan;
[2] Department of Respiratory Medicine, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan.

SUMMARY The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that struck in late 2019 and early 2020 is a serious threat to human health. Since there are no approved drugs that satisfactorily treat this condition, all efforts at drug design and/or clinical trials are warranted and reasonable. Drug repurposing is a well-known strategy that seeks to deploy existing licensed drugs for newer indications and that provides the quickest possible transition from the bench to the bedside to meet therapeutic needs. At present, several existing licensed drugs such as chloroquine, hydroxychloroquine, methylprednisolone, dexamethasone, and remdesivir have been used because of their potential efficacy in inhibiting COVID-19. Recently, antibiotics such as tetracyclines and macrolides have been reported to be effective against COVID-19. A combination of tetracyclines and macrolides may be a potential treatment for COVID-19 because there are some differences in the mechanism of action of tetracyclines and macrolides.

Keywords tetracycline, macrolide, COVID-19, SARS-CoV-2

The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that struck in late 2019 and early 2020 is a serious threat to human health. Since there are no approved drugs that satisfactorily treat this condition, all efforts at drug design and/or clinical trials are warranted and reasonable. Drug repurposing is a well-known strategy that seeks to deploy existing licensed drugs for newer indications and provides the quickest possible transition from the bench to the bedside to meet therapeutic needs. At present, several existing licensed drugs such as chloroquine, hydroxychloroquine, methylprednisolone, dexamethasone, and remdesivir have been used because of their potential efficacy in inhibiting COVID-19. Recently, antibiotics such as tetracyclines and macrolides have been reported to be effective against COVID-19.

Tetracyclines such as doxycycline, minocycline, and tetracycline are highly lipophilic antibiotics that are known to chelate zinc compounds on matrix metalloproteinases (MMPs). Several functions of SARS-CoV-2 are associated with the host MMP complex, including replication. Therefore, the zinc-chelating properties of tetracyclines may aid in inhibiting COVID-19 in humans, limiting the ability of SARS-CoV-2 to replicate within the host (*1,2*). Tetracycline is also reported to inhibit the binding of the SARS-CoV-2 spike protein to angiotensin-converting enzyme 2 (ACE2) (*3*). Thus, infection of cells by SARS-CoV-2 may be inhibited. Tetracyclines are effective in reducing the duration of ventilator support and intensive care unit stay from acute respiratory distress syndrome caused by COVID-19 (*3*). In addition, Yates *et al*. reported that doxycycline, at doses of 100-200 mg/day over 5-14 days, successfully treated 4 high-risk COVID-19-positive patients with pulmonary disease (*4*).

Macrolides such as erythromycin, clarithromycin, and azithromycin exhibit antibacterial activity, immunomodulatory action, and anti-inflammatory action. Lately, the antiviral action of macrolides has attracted considerable attention (*5*). Azithromycin accumulates within the lysosomes and increases their pH, resulting in lysosomal membrane disruption. Thus, viral replication is inhibited because SARS-CoV-2 replication depends on intact lysosomes (*5*). Moreover, azithromycin blocks the interaction points between SARS-CoV-2 and the ACE2 receptor, precluding SARS-CoV-2 from entering host cells (*5*).

Gautret *et al*. conducted a study that divided patients with COVID-19 into three groups: 6 patients with COVID-19 who were treated with hydroxychloroquine

(200 mg, 3 times per d, for 10 d) in combination with azithromycin (500 mg on day 1, followed by 250 mg per d for the next 4 d); 14 patients with COVID-19 who were treated with hydroxychloroquine alone; and 16 control patients with COVID-19. In these three groups, the patients' viral loads were assessed daily with a real-time reverse transcription polymerase chain reaction (PCR)-based analysis of nasopharyngeal swabs. Thus, 100% of the patients treated with hydroxychloroquine in combination with azithromycin were virologically cured on day 6. In contrast, 57.1% of the patients treated with hydroxychloroquine alone and 12.5% of the control group were virologically cured ($p < 0.001$). Moreover, 1 patient who was treated with hydroxychloroquine alone was still PCR-positive on day 6, but the patient was virologically cured by administration of azithromycin (*6*). Huang *et al*. found that clarithromycin was effective in the management of COVID-19 pneumonia within 6-12 days (*7*). Apart from the aforementioned macrolide antibiotics, ivermectin, a macrolide antiparasitic agent, is also an inhibitor of SARS-CoV-2, with a single treatment causing a ~5,000-fold reduction in the virus at 48 h in cell culture (*8*). Moreover, Ahmed *et al*. reported that a 5-day course of ivermectin reduced the duration of COVID-19 (*9*).

As an example of tetracyclines and macrolides in the treatment of COVID-19, a combination of doxycycline and ivermectin reduced the time to recovery and the percentage of patients who progressed to a more advanced stage of the disease; in addition, this treatment reduced the mortality rate in patients with severe COVID-19 from 22.72% to 0% compared to standard care with azithromycin (*10*). A combination of tetracyclines and macrolides, such as doxycycline and azithromycin, may be used because there are some differences in the mechanism of action of tetracyclines and macrolides. Moreover, this combined therapy may prevent the emergence of drug-resistant SARS-CoV-2. Taken together, the findings above indicate that a combination of tetracyclines and macrolides may be a potential therapeutic regimen for COVID-19 and open the door for an international strategy to fight this emerging viral infection.

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**Address correspondence to:*
Masashi Ohe, Department of Internal Medicine, JCHO Hokkaido Hospital, 1-8-3-18 Nakanoshima, Toyohira-ku, Sapporo 062-8618, Japan.
E-mail: oektsp1218@sweet.ocn.ne.jp

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Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

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BioScience Trends
Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@biosciencetrends.com