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Complete List of Authors:	Ohira, Masahiro; Hiroshima University Hospital, Kobayashi, Tsuyoshi; Hiroshima University Hospital Tanaka, Yuka; Hiroshima University Hospital Imaoka, Yuki; Hiroshima University Hospital Sato, Koki; Hiroshima University Hospital Imaoka, Koki; Hiroshima University Hospital Nakano, Ryosuke; Hiroshima University Hospital Doskali, Marlen; Hiroshima University Hospital Piao, Jinlian; Hiroshima University Hospital Nakamura, Mayuna; Hiroshima University Hospital Yoshida, Tetsumi; Hiroshima University Hospital Ichinohe, Tatsuo; Hiroshima University Hospital Kawano, Reo; Hiroshima University Hospital Yoshimura, Kenichi; Hiroshima University Hospital Ueda, Keiko; Hiroshima University Hospital Tamura, Natsuko; Hiroshima University Hospital Hirata, Taizo; Hiroshima University Hospital Imamura, Michio; Hiroshima University Hospital Aikata, Hiroshi; Hiroshima University Hospital Tanimine, Naoki; Hiroshima University Hospital Kuroda, Shintaro; Hiroshima University Hospital Tahara, Hiroyuki; Hiroshima University Hospital Ide, Kentaro; Hiroshima University Hospital Ohdan, Hideki; Hiroshima University Hospital
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Adoptive immunotherapy with natural killer cells from peripheral blood CD34$^+$ stem cells to prevent hepatocellular carcinoma recurrence after curative hepatectomy: A study protocol for an open-label, single-arm phase I study

Masahiro Ohira[1,2], Tsuyoshi Kobayashi[1], Yuka Tanaka[1], Yuki Imaoka[1], Koki Sato[1], Koki Imaoka[1], Ryosuke Nakano[1], Marlen Doskali[1], Jinlian Piao [1], Mayuna Nakamura[1], Tetsumi Yoshida[3], Tatsuo Ichinohe[3], Reo Kawano[4], Kenichi Yoshimura[2,4], Keiko Ueda[4], Natsuko Tamura[4], Taizo Hirata[4], Michio Imamura[5], Hiroshi Aikata[5], Naoki Tanimine[1], Shintaro Kuroda[1], Hiroyuki Tahara[1], Kentaro Ide[1], Hideki Ohdan[1,*]

Author affiliations

[1]*Department of Gastroenterological and Transplant Surgery, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan*

[2] *Medical Center for Translational and Clinical Research, Hiroshima University Hospital, Hiroshima, Japan*

[3]*Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan*

[4] *Clinical Research Center in Hiroshima, Hiroshima University Hospital, Hiroshima, Japan*

[5]*Department of Gastroenterology and Metabolism, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan*

[*] Correspondence and reprint requests to Hideki Ohdan, M.D., Ph.D., Department of Gastroenterological and Transplant Surgery, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. E-mail: hohdan@hiroshima-u.ac.jp Tel: 81-82-257-5220

Word count: 1989 words

Abstract

Introduction:

Hepatocellular carcinoma (HCC) remains a major clinical problem as more than half of these cases recur after radical resection. Natural killer (NK) cells are at the forefront of the innate immune system and attack microcarcinomas and circulating tumor cells. The objective of this study was to evaluate the feasibility and toxicity of peripheral blood $CD34^+$ stem cell–derived NK cell infusion after radical hepatectomy for HCC.

Methods and analysis:

This is an open-label, single-arm, single-center phase I study. Patients who have undergone initial hepatectomy for HCC with three or more risk factors for recurrence (≥ 10 ng/mL of AFP, ≥ 360 mAU/mL of PIVKA-II, multiple tumors, and ≥ 3 peripheral blood circulating tumor cells) will be enrolled and be treated with three peripheral blood $CD34^+$ stem cell–derived NK cell infusions every 3 months. The primary endpoint will be safety assessment including the type and severity of adverse events, frequency of occurrence, and duration of occurrence. The secondary endpoints will include survival, effect of immune response, and clinical laboratory test results.

Ethics and dissemination:

Ethical approval of the trial was obtained from the Certified Committee for Regenerative Medicine Hiroshima University in Japan. The trial results will be shared with the scientific community at international conferences and by publication in a peer-reviewed journal.

Trial registration number

jRCTb060200020; Pre-result

https://jrct.niph.go.jp/latest-detail/jRCTb060200020.

Keywords: immunotherapy, NK cell, hepatectomy, hepatocellular carcinoma, $CD34^+$ stem cells

Strengths and limitations of this study

- Currently, there are few adjuvant therapies after hepatectomy for advanced HCC.
- This is the first study with peripheral blood $CD34^+$ stem cell derived NK cells for the patients who received initial hepatectomy for advanced HCC.
- This phase I trial is not powered for efficacy endpoints and is therefore only suited to gather preliminary efficacy data.

- Based on the results of this study, future phase II study will be designed to investigate the efficacy of this approach and to improve the outcomes of advanced HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of cancer death worldwide [1]. Although HCC can be cured by hepatic resection, HCC recurrence occurs in almost 70% of patients within 5 years [2]. Furthermore, no standard adjuvant therapy has yet been established with proven efficacy in preventing recurrence.

Natural killer (NK) cells are the first defense of the cancer immune system and can kill target cells without prior sensitization based on inhibitory receptor recognition (missing–self hypothesis) and activated receptor recognition [3]. NK cell activity is significantly decreased in patients with liver cirrhosis [4], after hepatectomy [5], and during chemotherapy [6]. In our previous studies, liver mononuclear cells derived from donor liver perfusate contained many NK cells that have vigorous cytotoxicity against hepatoma cells; these NK cells express tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) after in vitro stimulation [4, 5, 7]. We have undertaken a pilot clinical trial for an adoptive immunotherapy approach, using lymphocytes extracted from the liver allograft perfusate, which includes an abundance of NK cells that mount an anti-HCC response, in liver transplant recipients with HCC[8, 9]. However, since liver-derived NK cells can only be obtained from liver transplant donors, we developed a procedure to generate hepatic endogenous NK cell-like cells from peripheral blood $CD34^+$ stem cells. This clinical phase I study aims to determine the feasibility of adoptive immunotherapy using peripheral blood $CD34^+$ stem cell–derived NK cells to prevent HCC recurrence after curative hepatectomy.

METHODS AND ANALYSIS

Study design

This clinical study is a Phase I trial of an immunostimulatory therapy using peripheral blood $CD34^+$ stem cell–derived NK cells to prevent recurrence of HCC after liver resection in high-risk liver cancer patients. The primary endpoint of this study will be safety, and it will be conducted as an open-label study to collect more safety information when NK therapy is administered. The study design is showed in Figure 1.

Inclusion criteria

Inclusion criteria were patients

1) who underwent initial hepatectomy for HCC;

2) aged between 20 and 79 years;

3) with hemoglobin level of 10g/dL or greater and platelets of 100,000 /uL or greater;

4) who have appropriate vessels for apheresis or who agree to catheter insertion in case of inappropriate vessels;

5) who have three or more risk factors of: AFP > 10 ng/mL, PIVKA-II > 360mAU/mL, multiple tumors, and circulating tumor cells > 3 in the peripheral blood;

6) with performance status of 0 or 1;

7) with pathologically no tumor residue (R0); and

8) who have signed written informed consent.

Exclusion criteria

Exclusion criteria were patients

1) who have malignant disease other than HCC;

2) are on steroids or immunosuppressive drug therapy;

3) who have previously undergone hepatectomy;

4) are not eligible for recombinant human granulocyte colony stimulating factor (rhG-CSF) or apheresis;

i) with allergy to rhG-CSF or are pregnant or possibly pregnant,

ii) with a history of coronary artery disease or cerebrovascular disease within the past 6 months,

iii) with heart disease (EF < 25%), lung disease, or renal disease requiring treatment,

iv) with neurological disorders,

v) with suspected myeloproliferative disorders such as leukocytosis and thrombocytosis,

vi) with leukocytes less than 3,000 μL/mL or more than 10,000 μL/mL, and

vii) with a history of interstitial pneumonia;

5) who have been hospitalized more than 6 weeks after surgery due to postoperative complications; and

6) deemed by researchers to be inappropriate participants.

Concomitant treatment with anticancer and immunosuppressive agents will not be used. However, the use of steroids as a pretreatment for cell administration and the use of immunosuppressive agents for the treatment of adverse events (except for prophylactic measures for the occurrence of adverse events) will not be stopped.

Sample size calculation

There were 37 patients in a recurrence risk group among 132 liver resections for initial liver cancer performed between January 2015 and December 2018. If about 30% of the patients would participate in the clinical trial, this would be 12 cases over 3 years. Considering the dropout cases at the time of enrollment, the target number of patients was set at 10.

Treatment

One to three months after the initial hepatectomy for HCC, patients received rhG-CSF (Filgrastim® syringe, Kyowa Kirin) 400 μg/m^2 subcutaneously once daily for 4–6 days, and peripheral blood CD34$^+$ stem cell collection by apheresis was performed on the day of the last Filgrastim® syringe administration. Lymphocyte fractions were extracted from the peripheral blood CD34$^+$ stem cell-containing solution

using the specific gravity centrifugation method. Lymphocytes derived from $CD34^+$ stem cells in the peripheral blood were cultured with GMP-grade human Interleukin (IL)-7 (Miltenyi Biotec), GMP-grade human IL-15 (Miltenyi Biotec), GMP-grade human stem cell factor (Miltenyi Biotec), and GMP-grade human Fms-like tyrosine kinase 3 ligand (Miltenyi Biotec) (all at a final concentration of 20 ng/mL), and human AB serum (5% final concentration). The culture method is involved adding the medium every 3 days and changing the medium every 7 days for a total of 17 days.

Three days prior to cell administration (day 14), GMP-grade human IL-12 (Miltenyi Biotec) was added to the cell culture medium at a final concentration of 5 ng/mL to activate NK cells. One day prior to cell administration (day 16), GMP-grade human IL-2 (Immunes, Kyowa Pharm) was added to the medium at a final concentration of 100 IU/mL. On the day of treatment, the cell suspension was collected, centrifuged, washed with saline, and then suspended in albumin-containing saline and administered intravenously (Figure 2). Protocol treatment is defined as from apheresis to the completion of peripheral blood $CD34^+$ stem cell–derived activated NK cell administration. Patients will receive this protocol treatment every three months for a total of three times.

Follow-up and assessment of efficacy

The primary endpoint of the study will be to assess the safety of the treatment (type and severity of adverse events, frequency of occurrence, and duration of occurrence). The criteria for serious adverse

events will be as follows: hematologic toxicity (anemia, leukopenia, leukocytosis, and thrombocytopenia), hepatic function abnormalities (elevated AST, ALT, ALP, and T-Bil), electrolyte abnormalities (hyponatremia, hypokalemia, hypokalemia, hypercalcemia, hyperkalemia, and hypercalcemia), non-hematologic toxicity (general malaise, headache, and fever), anorexia, nausea, diarrhea, constipation, laryngeal edema, mucositis, dyspnea, hypoxia, pulmonary edema, neuropathy, skin rash, allergic reaction, cytokine release syndrome, and hypotension. The secondary endpoints will be the following: 1) overall survival and recurrence-free survival (1 and 3 years after surgery), 2) effect on immune response: evaluation of peripheral blood NK cell activity, 3) adverse event assessment by cell dose, 4) vital signs, and 5) clinical laboratory test results.

Immunological assessment

All flow cytometry analyses will be performed on a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). To detect the surface phenotype, leukocytes will be stained with the following monoclonal antibodies: against CD3, TRAIL, NKG2D, CD69, CD226, and CD56. The data will be analyzed using FlowJo software (Tree Star, Inc. Ashland, OR). A ^{51}Cr-release assay will be performed as previously described [4], using HepG2 tumor cells (ATCC) as targets. Briefly, ^{51}Cr-labeled target tumor cells will be added for 4 hours at 37°C to effector cells in round-bottomed 96-well microtiter plates (BD Biosciences, Discovery Labware). The percentage of specific ^{51}Cr release will be calculated as

follows: % cytotoxicity = [(cpm of experimental release – cpm of spontaneous release)/(cpm of maximum release – cpm of spontaneous release)] × 100. All assays will be performed in triplicate.

Safe evaluation and reporting of adverse effects

After cell administration, the investigator will record all adverse events (AEs), whether or not considering to be related to cell administration. In the event of serious complications related to prolonged hospitalization or death, the investigator will promptly report to the Committee for Regenerative Medicine and the Ministry of Health, Labor and Welfare. The principal investigator or sub-investigator will discontinue or suspend clinical research on the subject in the following cases: 1) If a positive result is found in the infectious disease test of the culture test material; 2) Protocol treatment is no longer possible; 3) Subject requests withdrawal of consent for participation in clinical research; or 4) The occurrence of an adverse event is recognized, and the principal investigator judges that it is difficult to continue clinical research.

Statistics

The primary objective will be to determine the safety of this treatment. Safety assessment will include observation and recording of any grade of AEs according to the latest version of the NCI-CTC. As a rule, for items observed as continuous values, the number of examples, number of missing examples, mean (median), standard deviation (quartiles), and range (minimum-maximum) will be calculated as summary

statistics. For items observed as discrete values, the number of examples in each category and their percentages will be calculated as summary statistics. Statistical tests for the main analysis will be performed at a 5% significance level (two-sided).

Patient and Public Involvement

No patient involved.

ETHICS AND DISSEMINATION

This trial will be conducted in conformance with the principles of the 'Declaration of Helsinki'. All patients must be given written informed consent to a member of the study team before inclusion in this study. The protocol is approved by the Certified Committee for Regenerative Medicine Hiroshima University, Japan and has been prospectively registered in Japan Registry of Clinical Trials (jRCTb060200020).

A summary of the results will be provided the competent authority and the Certified Committee for Regenerative Medicine Hiroshima University within 1 year after the end of the trial. All subject records will be retained for 30 years following completion, termination, or discontinuation of the clinical investigation. The results of the clinical trial will be published in a peer-reviewed journal.

An electronic case report form is made using REDCap Electronic Data Capture, where all data on patient eligibility, treatment cycles and clinical parameters will be collected by trained staff-members in Hiroshima University Hospital. The clinical trial will be monitored approximately twice a year by an independent monitor.

DISCUSSION

This trial will investigate whether adoptive immunotherapy using peripheral blood $CD34^+$ stem cell–derived NK cells can be safely administered to HCC patients after curative resection. The primary endpoint will be to assess the safety of this treatment protocol. The secondary endpoints will be survival, recurrence-free survival, immunological assessment, side effects, and laboratory data analysis.

There are several methods of NK cell-based cell therapy, which can be categorized into those using peripheral blood primary NK cells, apheresis products, and umbilical cord blood [10]. Peripheral blood NK cells are easy to use and have been used for a long time, but their cellular differentiation is mature, it is difficult to obtain further enhancement of their function and their life span is short. In addition, NK cell activity in patients with liver cirrhosis and HCC is already exhausted [11-13], which is not desirable for cell therapy. Conversely, the method of developing NK cells from hematopoietic stem cells by apheresis is not affected by the primary disease, and since NK cells are manufactured from undifferentiated cells, they can be expected to be active in the body. For a combination of others, T cells need to be removed because

of the risk of graft-versus-host-disease and the high possibility of rapid elimination of the administered NK cells [14].

Phase I clinical trials of cell products stimulated and cultured from autologous or allogeneic peripheral blood lymphocytes have been conducted in lymphoma, breast cancer [15], gastric cancer [16], gastrointestinal cancer [17], non–small cell lung cancer [18], and other cancer types, and the safety of cell therapy has been uniformly reported, although the formulation process has differed. However, phase II efficacy studies have not shown efficacy against these cancer types [14]. Under these circumstances, postoperative adjuvant therapy with autologous peripheral blood–derived lymphocytes, as adjuvant therapy after curative resection for HCC has been shown to be effective in preventing recurrence in randomized controlled trials in Japan and overseas [19, 20]. These studies showed prolonged recurrence-free survival but not overall survival. Recently, however, multiple doses of autologous peripheral blood–derived activated lymphocytes were reported to prolong recurrence-free survival and overall survival as adjuvant therapy after local treatment, including surgery [21]. From these studies, HCC would be a cancer type that is expected to benefit from cell therapy.

We will thus analyze the safety and potential anti-tumor effects of the therapy in this clinical study. We expect that these findings will lead to future phase II/III trials.

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Contributors

MO participated in the trial design, performed the NK therapy, and prepared the manuscript. YI, KS, KI, MD, MN, RN, and YT prepared the study protocol, and performed NK therapy. HM, NT, MH, SK, HT, KI, and TK assisted in preparing the study protocol and performed the surgical procedures. YT, MD, KI, JP, and MN assisted in the preparation of the study protocol and conducted immunological analysis. TY and TI assisted in apheresis preparation. KK and KY assisted in the preparation of the analysis protocol. DS, KU, NT, TH, MI, and HA prepared the study protocol. HO designed the trial, prepared the study protocol, and conducted the correspondence. All authors have read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Patient consent for publication

Not applicable

Provenance and peer review

Not commissioned, externally peer reviewed.

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FIGURE LEGENDS

Figure 1. Study design.

Figure 2. Cell processing protocol.

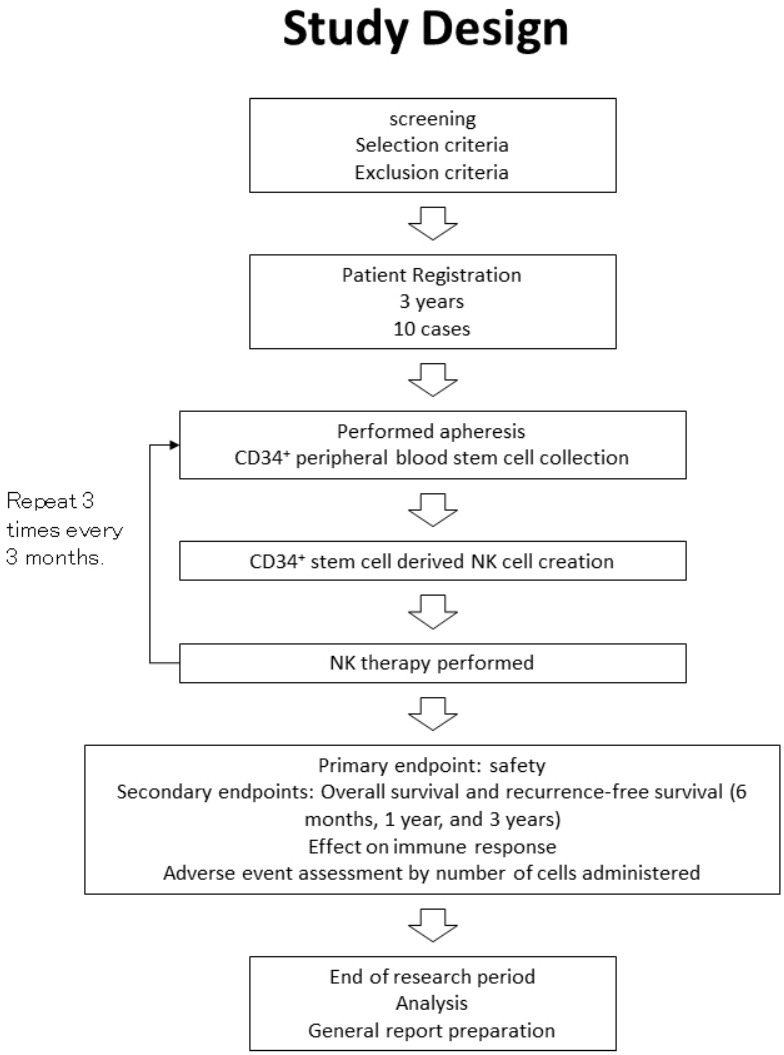

Fig. 1

Figure 1

190x254mm (96 x 96 DPI)

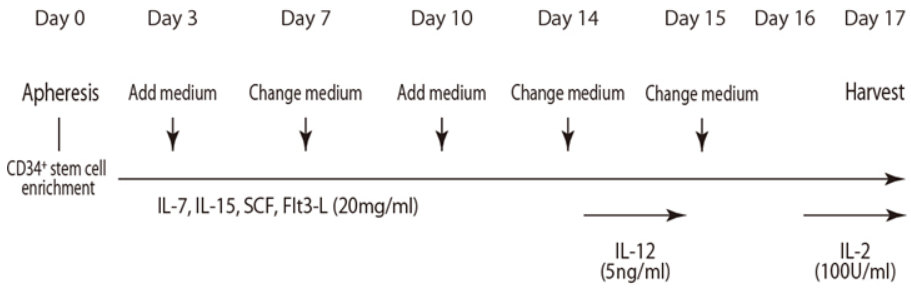

Fig. 2

Figure 2

190x254mm (96 x 96 DPI)

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRITreporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Gøtzsche PC, Altman DG, Mann H, Berlin J, Dickersin K, Hróbjartsson A, Schulz KF, Parulekar WR, Krleža-Jerić K, Laupacis A, Moher D. SPIRIT 2013 Explanation and Elaboration: Guidance for protocols of clinical trials. BMJ. 2013;346:e7586

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	3
Protocol version	#3	Date and version identifier	N/A
Funding	#4	Sources and types of financial, material, and other support	16
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	15

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	16
Roles and responsibilities: sponsor and funder	#5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	16
Roles and responsibilities: committees	#5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	16
Introduction			
Background and rationale	#6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	5
Objectives	#7	Specific objectives or hypotheses	5
Trial design	#8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	#9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	6
Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will	6

		perform the interventions (eg, surgeons, psychotherapists)	
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	8
Interventions: modifications	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	8
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	8
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	8
Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	6
Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Figure 1
Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	8
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	8
Methods: Assignment of interventions (for controlled trials)			
Allocation: sequence generation	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	N/A

Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	N/A
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	N/A
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	N/A
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A
Methods: Data collection, management, and analysis			
Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9,10
Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	9,10
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	11
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	11
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	10

Statistics: analysis population and missing data	#20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	11,12
Methods: Monitoring			
Data monitoring: formal committee	#21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	12
Data monitoring: interim analysis	#21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	12
Harms	#22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	12
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	12
Protocol amendments	#25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC / IRBs, trial participants, trial registries, journals, regulators)	11
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	12
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	12
Confidentiality	#27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	12

Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	16
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	12
Ancillary and post trial care	#30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	12
Dissemination policy: trial results	#31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	12
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	12
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	12
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	N/A
Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	12

BMJ Open

Adoptive immunotherapy with natural killer cells from peripheral blood CD34+ stem cells to prevent hepatocellular carcinoma recurrence after curative hepatectomy: A study protocol for an open-label, single-arm phase I study

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Keywords:	IMMUNOLOGY, Hepatobiliary surgery < SURGERY, SURGERY

Adoptive immunotherapy with natural killer cells from peripheral blood CD34^{+} stem cells to prevent hepatocellular carcinoma recurrence after curative hepatectomy: A study protocol for an open-label, single-arm phase I study

Masahiro Ohira[1,2], Tsuyoshi Kobayashi[1], Yuka Tanaka[1], Yuki Imaoka[1], Koki Sato[1], Koki Imaoka[1], Ryosuke Nakano[1], Marlen Doskali[1], Jinlian Piao [1], Mayuna Nakamura[1], Tetsumi Yoshida[3], Tatsuo Ichinohe[3], Reo Kawano[4], Kenichi Yoshimura[2,4], Keiko Ueda[4], Natsuko Tamura[4], Taizo Hirata[4], Michio Imamura[5], Hiroshi Aikata[5], Naoki Tanimine[1], Shintaro Kuroda[1], Hiroyuki Tahara[1], Kentaro Ide[1], Hideki Ohdan[1,*]

Author affiliations

[1]*Department of Gastroenterological and Transplant Surgery, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan*

[2] *Medical Center for Translational and Clinical Research, Hiroshima University Hospital, Hiroshima, Japan*

[3]*Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan*

[4] *Clinical Research Center in Hiroshima, Hiroshima University Hospital, Hiroshima, Japan*

[5]*Department of Gastroenterology and Metabolism, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan*

[*] Correspondence and reprint requests to Hideki Ohdan, M.D., Ph.D., Department of Gastroenterological and Transplant Surgery, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. E-mail: hohdan@hiroshima-u.ac.jp Tel: 81-82-257-5220

Word count: 1989 words

Abstract

Introduction:

Hepatocellular carcinoma (HCC) remains a major clinical problem as more than half of these cases recur after radical resection. Natural killer (NK) cells are at the forefront of the innate immune system and attack microcarcinomas and circulating tumor cells. The objective of this study was to evaluate the feasibility and toxicity of peripheral blood $CD34^+$ stem cell–derived NK cell infusion after radical hepatectomy for HCC.

Methods and analysis:

This is an open-label, single-arm, single-center phase I study. Patients who have undergone initial hepatectomy for HCC with three or more risk factors for recurrence (≥ 10 ng/mL of AFP, ≥ 360 mAU/mL of PIVKA-II, multiple tumors, and ≥ 3 peripheral blood circulating tumor cells) will be enrolled and be treated with three peripheral blood $CD34^+$ stem cell–derived NK cell infusions every 3 months. The primary endpoint will be safety assessment including the type and severity of adverse events, frequency of occurrence, and duration of occurrence. The secondary endpoints will include survival, effect of immune response, and clinical laboratory test results.

Ethics and dissemination:

Ethical approval of the trial was obtained from the Certified Committee for Regenerative Medicine Hiroshima University in Japan. The trial results will be shared with the scientific community at international conferences and by publication in a peer-reviewed journal.

Trial registration number

jRCTb060200020; Pre-result

https://jrct.niph.go.jp/latest-detail/jRCTb060200020.

Keywords: immunotherapy, NK cell, hepatectomy, hepatocellular carcinoma, $CD34^+$ stem cells

Strengths and limitations of this study

- G-CSF administration and apheresis must be performed to yield CD34-derived stem cells.
- Activated NK cells are created from CD34-positive stem cells using cytokines.
- Patients receive this protocol treatment every three months for a total of three times.
- The feasibility study design with consequent small sample size does not allow for accurate survival analysis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of cancer death worldwide [1]. Although HCC can be cured by hepatic resection, HCC recurrence occurs in almost 70% of patients within 5 years [2]. Novel immune checkpoint inhibitor therapies for recurrent and advanced HCC have been substantial and reported to have good results[3]. However, no standard adjuvant therapy has yet been established with proven efficacy in preventing recurrence.

Natural killer (NK) cells are the first defense of the cancer immune system and can kill target cells without prior sensitization based on inhibitory receptor recognition (missing–self hypothesis) and activated receptor recognition [4]. NK cell activity is significantly decreased in patients with liver cirrhosis [5], after hepatectomy [6], and during chemotherapy [7]. In our previous studies, liver mononuclear cells derived from donor liver perfusate contained many NK cells that have vigorous cytotoxicity against hepatoma cells; these NK cells express tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) after in vitro stimulation [5, 6, 8]. We have undertaken a pilot clinical trial for an adoptive immunotherapy approach, using lymphocytes extracted from the liver allograft perfusate, which includes an abundance of NK cells that mount an anti-HCC response, in liver transplant recipients with HCC[9, 10]. However, since liver-derived NK cells can only be obtained from liver transplant donors, we developed a procedure to generate hepatic endogenous NK cell-like cells from peripheral blood $CD34^{+}$ stem cells. This clinical phase I study aims to determine the feasibility of adoptive

immunotherapy using peripheral blood CD34+ stem cell–derived NK cells to prevent HCC recurrence after curative hepatectomy.

METHODS AND ANALYSIS

Study design

This clinical study is a Phase I trial of an immunostimulatory therapy using peripheral blood CD34+ stem cell–derived NK cells to prevent recurrence of HCC after liver resection in high-risk liver cancer patients. The primary endpoint of this study will be safety, and it will be conducted as an open-label study to collect more safety information when NK therapy is administered. The study design is showed in Figure 1. Patients have been enrolled since November 2021. The study is scheduled to run until March 2026.

Inclusion criteria

Inclusion criteria were patients

1) who underwent initial hepatectomy for HCC;

2) aged between 20 and 79 years;

3) with hemoglobin level of 10g/dL or greater and platelets of 100,000 /uL or greater;

4) who have appropriate vessels for apheresis or who agree to catheter insertion in case of inappropriate vessels;

5) who have three or more risk factors of: AFP > 10 ng/mL, PIVKA-II > 360mAU/mL, multiple tumors, and circulating tumor cells > 3 in the peripheral blood;

6) with performance status of 0 or 1;

7) with pathologically no tumor residue (R0); and

8) who have signed written informed consent.

Exclusion criteria

Exclusion criteria were patients

1) who have malignant disease other than HCC;

2) are on steroids or immunosuppressive drug therapy;

3) who have previously undergone hepatectomy;

4) are not eligible for recombinant human granulocyte colony stimulating factor (rhG-CSF) or apheresis;

i) with allergy to rhG-CSF or are pregnant or possibly pregnant,

ii) with a history of coronary artery disease or cerebrovascular disease within the past 6 months,

iii) with heart disease (EF < 25%), lung disease, or renal disease requiring treatment,

iv) with neurological disorders,

v) with suspected myeloproliferative disorders such as leukocytosis and thrombocytosis,

vi) with leukocytes less than 3,000 μL/mL or more than 10,000 μL/mL, and

vii) with a history of interstitial pneumonia;

5) who have been hospitalized more than 6 weeks after surgery due to postoperative complications; and

6) deemed by researchers to be inappropriate participants.

Concomitant treatment with anticancer and immunosuppressive agents will not be used. However, the use of steroids as a pretreatment for cell administration and the use of immunosuppressive agents for the treatment of adverse events (except for prophylactic measures for the occurrence of adverse events) will not be stopped.

Sample size calculation

There were 37 patients in a recurrence risk group among 132 liver resections for initial liver cancer performed between January 2015 and December 2018. If about 30% of the patients would participate in the clinical trial, this would be 12 cases over 3 years. Considering the dropout cases at the time of enrollment, the target number of patients was set at 10.

Treatment

One to three months after the initial hepatectomy for HCC, patients received rhG-CSF (Filgrastim® syringe, Kyowa Kirin) 400 μg/m^2 subcutaneously once daily for 4–6 days, and peripheral blood CD34$^+$ stem cell collection by apheresis was performed on the day of the last Filgrastim® syringe administration. Lymphocyte fractions were extracted from the peripheral blood CD34$^+$ stem cell-containing solution using the specific gravity centrifugation method. Lymphocytes derived from CD34$^+$ stem cells in the peripheral blood were cultured with GMP-grade human Interleukin (IL)-7 (Miltenyi Biotec), GMP-grade human IL-15 (Miltenyi Biotec), GMP-grade human stem cell factor (Miltenyi Biotec), and GMP-grade human Fms-like tyrosine kinase 3 ligand (Miltenyi Biotec) (all at a final concentration of 20 ng/mL), and human AB serum (5% final concentration). The culture method is involved adding the medium every 3 days and changing the medium every 7 days for a total of 17 days.

Three days prior to cell administration (day 14), GMP-grade human IL-12 (Miltenyi Biotec) was added to the cell culture medium at a final concentration of 5 ng/mL to activate NK cells. One day prior to cell administration (day 16), GMP-grade human IL-2 (Immunes, Kyowa Pharm) was added to the medium at a final concentration of 100 IU/mL. On the day of treatment, the cell suspension was collected, centrifuged, washed with saline, and then suspended in albumin-containing saline and administered intravenously (Figure 2). Protocol treatment is defined as from apheresis to the completion of peripheral

blood $CD34^+$ stem cell–derived activated NK cell administration. Patients will receive this protocol treatment every three months for a total of three times.

Follow-up and assessment of efficacy

The primary endpoint of the study will be to assess the safety of the treatment (type and severity of adverse events, frequency of occurrence, and duration of occurrence). The criteria for serious adverse events will be as follows: hematologic toxicity (anemia, leukopenia, leukocytosis, and thrombocytopenia), hepatic function abnormalities (elevated AST, ALT, ALP, and T-Bil), electrolyte abnormalities (hyponatremia, hypokalemia, hypokalemia, hypercalcemia, hyperkalemia, and hypercalcemia), non-hematologic toxicity (general malaise, headache, and fever), anorexia, nausea, diarrhea, constipation, laryngeal edema, mucositis, dyspnea, hypoxia, pulmonary edema, neuropathy, skin rash, allergic reaction, cytokine release syndrome, and hypotension. The secondary endpoints will be the following: 1) overall survival and recurrence-free survival (1 and 3 years after surgery), 2) effect on immune response: evaluation of peripheral blood NK cell activity, 3) adverse event assessment by cell dose, 4) vital signs, and 5) clinical laboratory test results.

Immunological assessment

All flow cytometry analyses will be performed on a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). To detect the surface phenotype, leukocytes will be stained with the

following monoclonal antibodies: against CD3, TRAIL, NKG2D, CD69, CD226, and CD56. The data will be analyzed using FlowJo software (Tree Star, Inc. Ashland, OR). A ^{51}Cr-release assay will be performed as previously described [5], using HepG2 tumor cells (ATCC) as targets. Briefly, ^{51}Cr-labeled target tumor cells will be added for 4 hours at 37°C to effector cells in round-bottomed 96-well microtiter plates (BD Biosciences, Discovery Labware). The percentage of specific ^{51}Cr release will be calculated as follows: % cytotoxicity = [(cpm of experimental release – cpm of spontaneous release)/(cpm of maximum release – cpm of spontaneous release)] × 100. All assays will be performed in triplicate.

Safe evaluation and reporting of adverse effects

After cell administration, the investigator will record all adverse events (AEs), whether or not considering to be related to cell administration. In the event of serious complications related to prolonged hospitalization or death, the investigator will promptly report to the Committee for Regenerative Medicine and the Ministry of Health, Labor and Welfare. The principal investigator or sub-investigator will discontinue or suspend clinical research on the subject in the following cases: 1) If a positive result is found in the infectious disease test of the culture test material; 2) Protocol treatment is no longer possible; 3) Subject requests withdrawal of consent for participation in clinical research; or 4) The occurrence of an adverse event is recognized, and the principal investigator judges that it is difficult to continue clinical research. Our protocol specifies that a clinical trial will be terminated as follows; 1. Significant information regarding the quality, safety, or efficacy of the cellular conditioning is obtained. 2. When

it is deemed difficult to conduct the clinical research due to delays in case enrollment, frequent protocol deviations, or other reasons. 3. When it is determined that there is a problem with the safety of the protocol treatment based on the evaluation by the Committee for Regenerative Medicine. 4. If, as a result of the evaluation of relevant information obtained from sources other than this clinical research, such as papers and conference presentations, it is determined that there is a problem with the safety of the protocol treatment, or that the continuation of the clinical research is no longer meaningful.

Statistics

The primary objective will be to determine the safety of this treatment. Safety assessment will include observation and recording of any grade of AEs according to the latest version of the NCI-CTC. As a rule, for items observed as continuous values, the number of examples, number of missing examples, mean (median), standard deviation (quartiles), and range (minimum-maximum) will be calculated as summary statistics. For items observed as discrete values, the number of examples in each category and their percentages will be calculated as summary statistics. Statistical tests for the main analysis will be performed at a 5% significance level (two-sided).

Patient and Public Involvement

No patient involved.

ETHICS AND DISSEMINATION

This trial will be conducted in conformance with the principles of the 'Declaration of Helsinki'. All patients must be given written informed consent to a member of the study team before inclusion in this study. The protocol is approved by the Certified Committee for Regenerative Medicine Hiroshima University, Japan and has been prospectively registered in Japan Registry of Clinical Trials (jRCTb060200020).

A summary of the results will be provided the competent authority and the Certified Committee for Regenerative Medicine Hiroshima University within 1 year after the end of the trial. All subject records will be retained for 30 years following completion, termination, or discontinuation of the clinical investigation. The results of the clinical trial will be published in a peer-reviewed journal.

An electronic case report form is made using REDCap Electronic Data Capture, where all data on patient eligibility, treatment cycles and clinical parameters will be collected by trained staff-members in Hiroshima University Hospital. The clinical trial will be monitored approximately twice a year by an independent monitor.

DISCUSSION

This trial will investigate whether adoptive immunotherapy using peripheral blood $CD34^+$ stem cell–derived NK cells can be safely administered to HCC patients after curative resection. The primary

endpoint will be to assess the safety of this treatment protocol. The secondary endpoints will be survival, recurrence-free survival, immunological assessment, side effects, and laboratory data analysis.

There are several methods of NK cell-based cell therapy, which can be categorized into those using peripheral blood primary NK cells, apheresis products, and umbilical cord blood [11]. Peripheral blood NK cells are easy to use and have been used for a long time, but their cellular differentiation is mature, it is difficult to obtain further enhancement of their function and their life span is short. In addition, NK cell activity in patients with liver cirrhosis and HCC is already exhausted [12-14], which is not desirable for cell therapy. Conversely, the method of developing NK cells from hematopoietic stem cells by apheresis is not affected by the primary disease, and since NK cells are manufactured from undifferentiated cells, they can be expected to be active in the body. For a combination of others, T cells need to be removed because of the risk of graft-versus-host-disease and the high possibility of rapid elimination of the administered NK cells [15].

Phase I clinical trials of cell products stimulated and cultured from autologous or allogeneic peripheral blood lymphocytes have been conducted in lymphoma, breast cancer [16], gastric cancer [17], gastrointestinal cancer [18], non–small cell lung cancer [19], and other cancer types, and the safety of cell therapy has been uniformly reported, although the formulation process has differed. However, phase II efficacy studies have not shown efficacy against these cancer types [15]. Under these circumstances,

postoperative adjuvant therapy with autologous peripheral blood–derived lymphocytes, as adjuvant therapy after curative resection for HCC has been shown to be effective in preventing recurrence in randomized controlled trials in Japan and overseas [20, 21]. These studies showed prolonged recurrence-free survival but not overall survival. Recently, however, multiple doses of autologous peripheral blood–derived activated lymphocytes were reported to prolong recurrence-free survival and overall survival as adjuvant therapy after local treatment, including surgery [22]. From these studies, HCC would be a cancer type that is expected to benefit from cell therapy.

We will thus analyze the safety and potential anti-tumor effects of the therapy in this clinical study. We expect that these findings will lead to future phase II/III trials.

Acknowledgements

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Contributorship Statement

MO, TK, YK and HO conceived the idea of the study. RK, KY, KU, NT and TH developed the statistical analysis plan and conducted statistical analyses. YI, KS, KI, RN, MD, JP, and MN manufactured samples. TY, TI, MI, HA, NT, SK, HT, and KI contributed to the interpretation of the results. MO drafted the original manuscript. MO supervised the conduct of this study. All authors reviewed the manuscript draft and revised it critically on intellectual content. All authors approved the final version of the manuscript to be published.

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Competing interests

The authors declare that they have no competing interests.

Patient consent for publication

Not applicable

Provenance and peer review

Not commissioned, externally peer reviewed.

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FIGURE LEGENDS

Figure 1. Study design.

Figure 2. Cell processing protocol.

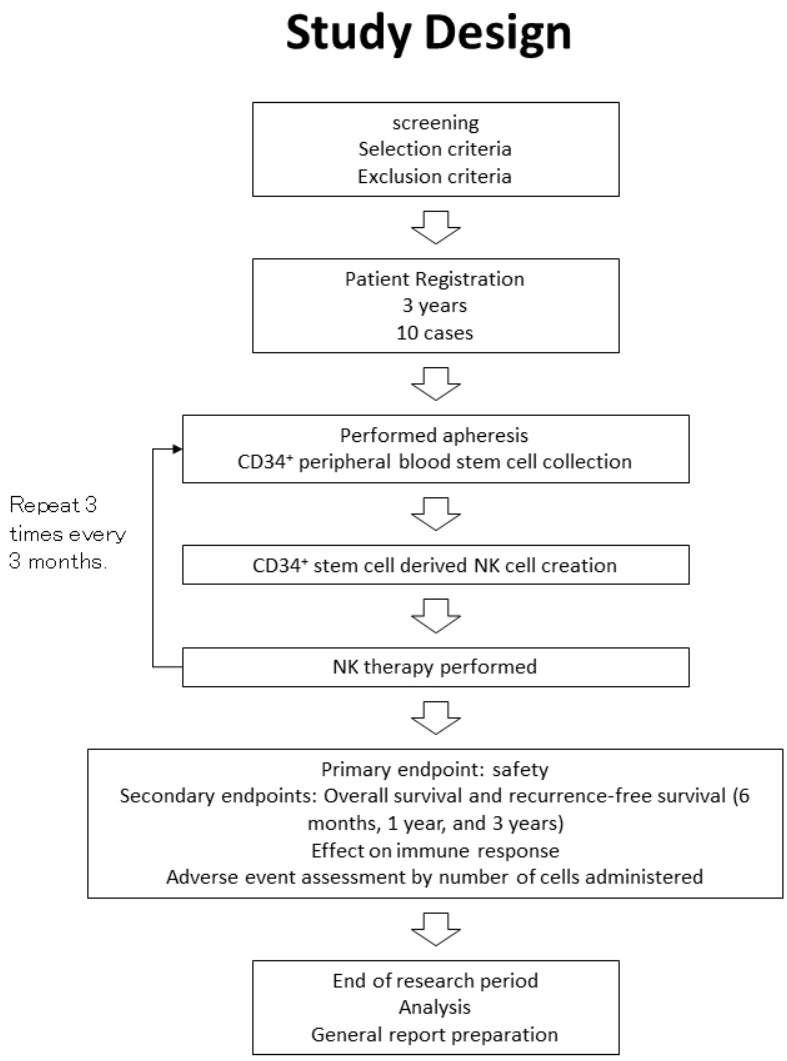

Fig. 1

Figure 1

190x254mm (96 x 96 DPI)

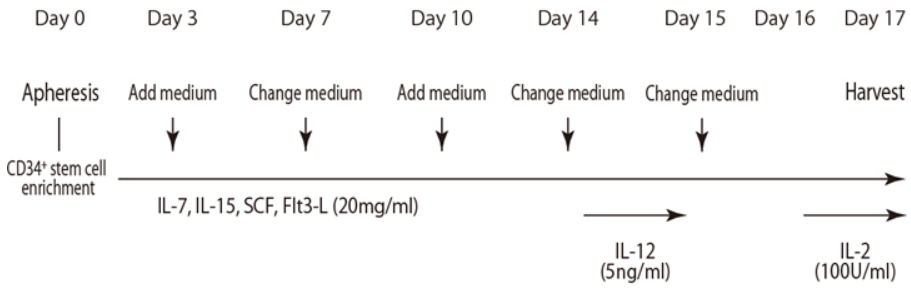

Fig. 2

Figure 2

190x254mm (96 x 96 DPI)

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRITreporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Gøtzsche PC, Altman DG, Mann H, Berlin J, Dickersin K, Hróbjartsson A, Schulz KF, Parulekar WR, Krleža-Jerić K, Laupacis A, Moher D. SPIRIT 2013 Explanation and Elaboration: Guidance for protocols of clinical trials. BMJ. 2013;346:e7586

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	3
Protocol version	#3	Date and version identifier	N/A
Funding	#4	Sources and types of financial, material, and other support	16
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	15

Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	16
Roles and responsibilities: sponsor and funder	#5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	16
Roles and responsibilities: committees	#5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	16
Introduction			
Background and rationale	#6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	5
Objectives	#7	Specific objectives or hypotheses	5
Trial design	#8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	#9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	6
Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will	6

		perform the interventions (eg, surgeons, psychotherapists)	
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	8
Interventions: modifications	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	8
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	8
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	8
Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	6
Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Figure 1
Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	8
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	8
Methods: Assignment of interventions (for controlled trials)			
Allocation: sequence generation	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	N/A

Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	N/A
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	N/A
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	N/A
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A
Methods: Data collection, management, and analysis			
Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9,10
Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	9,10
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	11
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	11
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	10

Statistics: analysis population and missing data	#20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	11,12
Methods: Monitoring			
Data monitoring: formal committee	#21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	12
Data monitoring: interim analysis	#21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	12
Harms	#22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	12
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	N/A
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	12
Protocol amendments	#25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC / IRBs, trial participants, trial registries, journals, regulators)	11
Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	12
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	12
Confidentiality	#27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	12

Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	16
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	12
Ancillary and post trial care	#30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	12
Dissemination policy: trial results	#31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	12
Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	12
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	12
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	N/A
Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	12