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Efficacy and safety of natural killer cells injection combined with XELOX chemotherapy in postoperative patients with stage III colorectal cancer in China: a prospective randomised controlled clinical trial study protocol

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ABSTRACT

Background Colorectal cancer (CRC) is the second most frequently diagnosed cancer and the fifth leading cause of cancer-related death in China. However, resistance to multiple chemotherapeutics after surgery leads to failure of the main therapy to CRC. Natural killer (NK) cells are innate cytotoxic lymphocytes that exhibit strong cytotoxic activity against tumour cells. NK cell-based therapy, either alone or in combination with chemotherapy, has achieved favourable results and holds promise for addressing recurrence and metastasis in CRC patients after surgery.

Methods and analysis This is a prospective, randomised controlled clinical trial to evaluate efficacy and safety of interleukin 2 activated NK cells injection combined with XELOX (capecitabine plus oxaliplatin)-based chemotherapy for postoperative CRC patients. Participants will be randomly divided into treatment group and control group, and every group includes 40 patients. The treatment group will also receive NK cells (5×10^9) with+XELOX-based chemotherapy, while the control group will receive only XELOX-based chemotherapy. This treatment will be repeated for eight cycles (6 months). The follow-up period lasts about 3 years, during which CEA, CA19-9, CA125, enhancement CT and colonoscopy will be conducted. The primary endpoints of this study are progression-free survival and overall survival, while the secondary endpoint is safety (number and severity of adverse events). Additionally, we aim to identify cancer stem cells in peripheral blood and predictive biomarkers (cytokines secreted by NK cells and activated markers of NK cells) that indicate patients who achieve an effective response.

Ethics and dissemination The study has been approved by the Clinical Research Ethics Committee of our hospital (approval number 2023LLSC006) and the Chinese Clinical Trials. It will be conducted in accordance with the Declaration of Helsinki. Written informed consent will be obtained from all participants. The study findings will be submitted to peer-reviewed journals for publication.

Trial registration number Chinese Clinical Trials Registry (ChiCTR2300075861).

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ The study adheres to the Standard Protocol Items: Recommendations for Interventional Trials statement recommendations, which ensures that the study is conducted with transparency and using high-quality standards.
⇒ The sample size calculation will ensure an early study termination if there is insufficient efficacy of the intervention.
⇒ This study will report adverse events in Chinese patients with postoperative colorectal cancer (CRC).
⇒ Long-term adverse events after adjuvant chemotherapy for CRC can be identified based on patient-reported outcomes.
⇒ The study is a single-centre design, limiting the generalisability of the study findings to a general population.

INTRODUCTION

Despite advances in screening tests, surgery, radiation therapy and adjuvant chemotherapy, 40% of colorectal cancer (CRC) patients still die from tumour recurrence and metastasis.[1–3] CRC is a diverse disease with varying mutational profiles and compositions of the tumour microenvironment (TME), microsatellite instability (MSI-H) occurs in 15% of CRC patients and is caused by a deficiency in DNA mismatch repair (dMMR), which inhibits the correction of replication errors. MSI-H is an important biomarker with prognostic and predictive value, particularly in early-stage CRC, as it is associated with improved overall survival (OS) and a lower chance of relapse.[4 5] dMMR CRC patients are sensitive to immune checkpoint blockade, leading to prolonged disease control in the metastatic setting[6]; and recently it has been shown that neoadjuvant immunotherapy

in dMMR cases raises the possibility of organ-sparing therapy (NICHE trials).[7] Additionally, studies have indicated that patients with stage III or IV CRC, have more microsatellite-stable (MSS) but only a few cases combined with BRAF mutation and do not respond well to chemotherapy. So there is a need for new clinical trials to identify therapeutic options.[8–10]

TME in CRC is a key factor in cancer progression, immune cell dysfunction, and resistance to immunotherapy. Several metabolic features of the TME, such as hypoxia, elevated levels of acidosis, increased extracellular matrix rigidity and high interstitial fluid pressure, have a negative impact on natural killer (NK) cell activity.[11] Studies have shown that low oxygen levels in the TME impair the function of NK cells by downregulating activating signals like NKG2D, NKp30 and CD16, thus limiting cytokine production, cytotoxicity and promoting metastasis.[12 13] The acidic TME acts as a critical barrier created by tumour cells against NK cells, contributing to metastasis, tumour progression, immune cell suppression and poor prognosis.[14] Targeting metabolism in CRC shows promise as a therapeutic approach to overcome immune suppression.[15] In the TME, the NKG2D receptor of NK cells is often blocked by various ligands (eg, MICA, MICB and ULBP1-6) that are upregulated in tumour cells due to abnormal cellular stress.[16] Preclinical and clinical data suggest that the efficacy of NK cell infusion against solid malignancies is hindered by several factors, including inadequate tumour infiltration, persistence/activation in the TME.[17 18] Recent evidence suggests that cancer stem cells (CSCs), which are a subpopulation of cells with stem cell-like characteristics, play a crucial role in various aspects of CRC including initiation, progression, relapse, metastasis.[19–22] They are resistant to radiation and chemotherapy drugs.[22 23] It has been observed that conventional treatments like surgery and chemotherapy primarily target the bulk of the tumour, but they often fail to eliminate CSCs. In contrast, alternative therapies such as interleukin 2 (IL-2) activated NK cells have demonstrated better efficacy in targeting and eliminating quiescent or non-proliferating CSCs compared with colon cells. These findings highlight the potential of therapeutic approaches beyond chemotherapy and surgery for effectively addressing CSCs in CRC.[24 25]

Early during tumour development, NK-cell migration into CRC tumour tissue is clearly impaired, which is a poor prognostic factor for CRC in these patients. Dysfunction of NK cells not only leads to the proliferation of tumour cells but also the formation of distant metastases.[26 27] Moreover, Stroma AReactive Invasion Front Areas in TME comes along with distinct immunological alterations, especially decrease of peripheral blood NK cells.[28] NK cells are an integral part of the innate immune system and play a crucial role in promoting cytotoxic activity against tumour or infected cells, independent of MHC recognition.[29] The activity of NK cells towards target cells is determined by the expression of activator/inhibitory receptors. The main activating NK receptors include non-HLA-specific natural cytotoxicity receptors (NKp46, NKp30 and NKp44, collectively known as NCRs), NKG2D and DNAM-1.[30] These activator receptors regulate the cytotoxic activity of NK cells. Two major subsets of peripheral blood NK cells have been identified based on the surface expression of CD56 and CD16.[31] CD56–CD16+ NK cells (90%–95% of total circulating NK cells) are responsible for cytotoxic activities, such as perforin and granzyme release, as well as mediating antibody-dependent cell-mediated cytotoxicity. On the other hand, CD56+CD16− NK cells (5%–10% of total circulating NK cells) are capable of producing Th1 cytokines, such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). In recent years, there has been a significant increase in research on NK cell-related immunotherapy. Signs of NK cell activation have been associated with improved clinical outcomes in various oncological settings, including acute myeloid leukaemia,[32 33] gastrointestinal stromal tumours[34] and breast cancer.[35 36] The latest developments in this field have primarily focused on cytokine supplements, monoclonal antibodies and genetic engineering of NK cells.[37–39] However, it is important to note that the current process of producing CAR-NK cells is complex and expensive, and the efficacy against solid tumours is still not satisfactory. Despite these challenges, NK cell-based therapy has shown promising results when used alone or in combination with other treatments, indicating its potential for widespread and effective use in treating malignancies.[40 41] In the peripheral blood of CRC patients, there was an observed increase in the percentage of CD16+ NK cells reduction. This decrease in CD16+ NK cells has been linked to shorter disease-free survival.[42] Recent technological advancements have enabled the use of ex vivo-expanded NK cells for adoptive immunotherapy.[43–45] It is possible that an increased presence of CD16+ NK cells is required for them to function as effector cells.

Surgery and chemotherapy are conventional treatments for CRC patients. Surgery is effective in removing cancer masses and the surrounding TME from the body.[46] Additionally, it aids in the elimination of circulating tumour cells and CSC by enhancing the activity of NK cells. When combining with chemotherapy, surgery can lead to more favourable outcomes for various types of cancer. The commonly used chemotherapy plans for CRC patients include mFOLFOX6, FOLFIRI and XELOX.[47] mFOLFOX6, FOLFIRI and XELOX as the first-line treatment have been advised by the Chinese protocol of diagnosis and treatment of CRC (2023 edition) established by the National Health Commission of the People's Republic of China. Previous studies have demonstrated the positive effects of chemotherapeutics on the immune system.[48 49] For example, research has shown that 5-fluorouracil can increase the expression of tumour antigens in colorectal and breast cancer cells.[50] Additionally, oxaliplatin has been found to induce immunogenic cell death in CRC cells, contributing to its effectiveness as a therapy for CRC patients.[51] Only one trial has been conducted on the

combination therapy of cellular immunotherapy (the use of autologous NK cells, γδT cells and cytokine-induced killer cells) and chemotherapy, which has shown promising results in promoting the survival time, reducing the recurrence rate and improving the immune status of CRC patients. Moreover, this combination therapy is considered safe and low-toxic, thereby improving the patient's tolerance to chemotherapy.[52]

In this particular study, XELOX was used as a treatment option, which may be equally effective as FOLFOX. The treatment was found to be safe, with no instances of grade 3 or higher haematological toxicity. There is no reported trial on the combination therapy of IL-2 activated NK cells and XELOX-based chemotherapy for postoperative CRC patients. Therefore, the objective of this study is to assess the feasibility and safety of this combination therapy in addressing recurrence and metastasis in CRC patients after surgery.

The internal mechanism of NK cells killing tumour stem cells involves the activation of cancer cell surface receptors including NCRs and CD16. This activation triggers the secretion of cytokines like TNF-α and IFN-γ by NK cells, resulting in an enhanced capability of NK cells to kill tumour cells.

METHODS AND ANALYSIS

Study design and setting

This study is a prospective, randomised clinical trial conducted in accordance with ethical guidelines for clinical studies. The objective of this study is to evaluate the efficacy and safety of NK cells injection combined with XELOX chemotherapy in postoperative CRC patients. Participants will be randomly assigned to either the treatment group or the control group, both of which will receive XELOX-based chemotherapy. In addition to chemotherapy, the treatment group will receive intravenous administration of NK cells (5×10^9) and 0.9% sodium chloride 100 mL, while the control group will only receive intravenous administration of 0.9% sodium chloride 100 mL. This treatment will be repeated for eight cycles over approximately 6 months. Tumour markers (CEA, CA19-9, CA125) will be examined every cycle, and enhancement CT scans will be performed every 6 months, with a follow-up period of 2.5 years. The study will investigate progression-free survival (PFS), OS and adverse effects. Additionally, cytokines (TNF-α, IFN-γ) secreted by NK cells and activated markers of NK cells from CRC patients will be tested. The flow diagram is presented in figure 1.

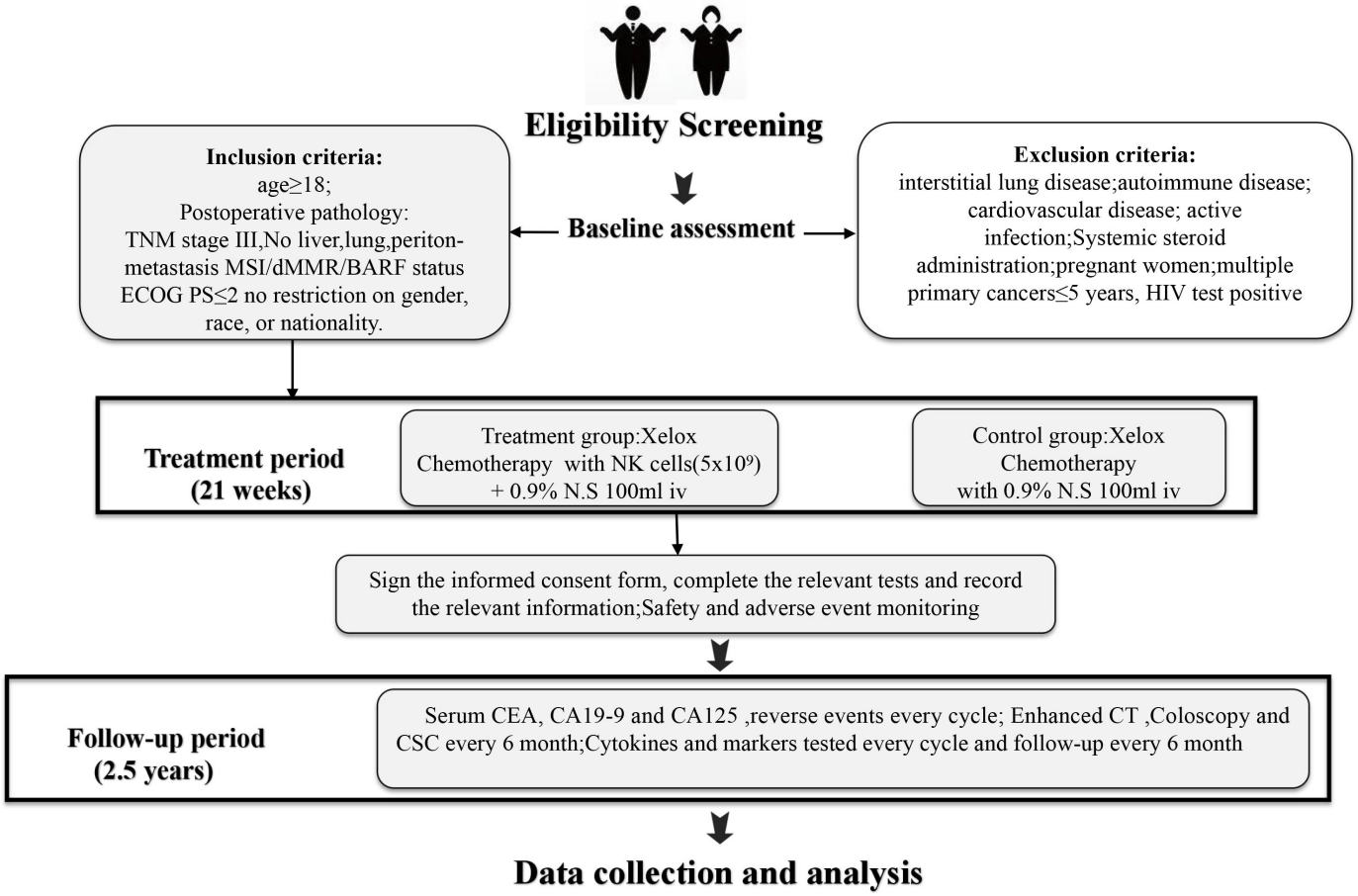

Figure 1 Flow diagram. CSC, cancer stem cells; dMMR, DNA mismatch repair; ECOG PS, Eastern Cooperative Oncology Group performance status; MSI, microsatellite instability; NK, natural killer; TNM, tumour, node, metastases.

Sample size and power analysis

The number of samples was determined based on a previous study, which found that the killing rate was 30% when NK cells were killing K562 cells at a ratio of 5:1. Another trial suggested that cell therapy using an infusion of autologous NK cells could reduce the relapse rate by up to 20% after 3 years.[52] We calculated the sample size needed to detect a difference in the percentage of autologous NK cells reducing the relapse rate, assuming an expected difference of 20%, an SD of 15%, an alpha level of 5% and a power of 80%. This resulted in a sample size of 30 for each group. Taking into account a dropout rate of 33%, the target sample size was set at 40 for each group.

Patient selection criteria

Inclusion criteria

All included cases (over 18 years old) must be confirmed as stage III CRC by pathology and MSI/MMR/BARF status by immunohistochemistry. Only MSS/pMMR stage III CRC tumours (including BRAF mutant or wild type) will be selected and all four MMR proteins be stained for immunohistochemistry. Liver, lung and peritoneal metastasis will not be considered. Additionally, patients must meet the following criteria: Eastern Cooperative Oncology Group performance status ≤2. There will be no restrictions based on gender, race or nationality. In the same original study, the chemotherapy regimen used for the control group of CRC patients is the same as that for the experimental group of CRC patients. The dosage and course of treatment are not limited.

Exclusion criteria

This study excluded patients with interstitial lung disease, autoimmune disease, clinically significant cardiovascular disease, active infection, systemic steroid administration, pregnant women, multiple primary cancers within the previous 5 years, positive HIV test result and any other conditions that made the patient unsuitable for this study.

Patient enrolment procedure

Each potential patient must be provided with a patient information sheet and obtain full written informed consent. This should include group-based information for CRC patients who are waiting for operations and are in the hospital. Additionally, individual information about each trial arm should be given (see online supplemental file 1 for participant consent form). To achieve the target sample size, strategies will be implemented to ensure adequate participant enrolment. Participants will be divided into a treatment group and a control group in a 1:1 ratio using a central network-based randomisation tool in this study. Independent statisticians will generate random sequences using SAS V.9.3 software. When the research assistant enters the patient information on the tablet computer, they will be assigned a random number and complete the randomisation process based on the assigned results. Throughout the study, all individuals, including researchers and patients, will be blinded to the group assignment until the end of the study.

Intervention and control arms

Patients with stage III CRC receive XELOX therapy, which consists of 130 mg/m^2 of oxaliplatin on day 1 and 1000 mg/m^2 of capecitabine two times a day on days 2–15, every 3 weeks. The treatment groups are divided into two: one group receiving NK cells plus XELOX and another group receiving only XELOX. In the NK cells plus XELOX group, a median of 5×10^9 NK cells cultured ex vivo with IL-2 will be injected intravenously into patients once every 3 weeks for 6 months (eight cycles). The NK cells will be infused on day 16 of the 21-day cycle. Dose reductions of capecitabine and oxaliplatin will be required for all grade 3 or 4 toxicities associated with the study medications.[53] The treatment will continue until disease progression, unacceptable toxicity or withdrawal of consent.

Data collection, management and analysis

Follow-up

Serum CEA, CA19-9, and CA125 levels will be obtained monthly in all patients. Abdominal contrast-enhanced CT and chest CT scans will be performed every 6 months. Further investigations will be carried out when clinically indicated or when tumour progression is suspected. OS will be defined as the period from the date of first treatment until death. Patients who do not experience an event will be censored on the date of last contact. PFS is defined as the period from the date of first treatment until the occurrence of an event (progressive disease, death or diagnosis of a second malignant neoplasm), whichever occurs first.

NK cell isolation and culture

For the study of human NK cells, blood samples were obtained from CRC patients and peripheral blood lymphocytes were isolated using Biocoll separating solution (Biochrom AG) through density gradient centrifugation. Enriched NK cells were then isolated from the separated peripheral blood mononuclear cells using the NK cell isolation kit (stem cell) following the manufacturer's instructions. The isolated NK cells were cultured overnight in RPMI 10% FBS and 1% penicillin/streptomycin. Subsequently, more than 1×10^6 harvested cells were cultured with IL-2 for 14 days. The purity of the isolated NK cells was assessed to be >95% using flow cytometry.

Flow cytometric analysis

Flow cytometry will be conducted to evaluate the surface expression levels of NCR ligands. The cells will be harvested and washed twice with cold phosphate-buffered saline. Subsequently, they will be incubated with the appropriate antibodies, following the manufacturer's instructions, for 30 min at 4°C in the dark. To assess the expression of NCR ligands, 100 µL of cell suspension will be added to NKG2D-APC, NKp30-PE and CD16-FITC, respectively. Another 100 µL of the cell suspension will be taken and NKp44-APC and Nkp46-Alexa Fluor 647

antibodies will be added. The mixture will be vortexed for 30 s and incubated in the dark for 15–20 min at room temperature. After incubation, the cells will be resuspended, centrifuged at 1500 rpm for 5 min, and this process will be repeated 1–2 times. Flow cytometric analysis will be performed using FACS Canto II (BD Biosciences) and analysed with CellQuest V.3.1.

Cytokines quantitation by ELISA

For cytokine release measurement, the supernatants of NK cells (the samples were stored at −70°C until they were subjected to ELISA) were collected and centrifuged at 1500× *g* and 4°C for 10 min. They were then incubated on ice and centrifuged at 13 000× *g* and 4°C for 10 min. The IL-6, IFN-γ and TNF-α levels were quantified using an ELISA kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. The amounts of these cytokines were expressed as ng/mL of NK cell media.

Detection of CSCs

CSCs detection will be performed on the blood of CRC patients as described elsewhere. Briefly, $CD44^+$ cells will be isolated using anti-CD44 microbeads (Miltenyi Biotec) following the manufacturer's instructions. The freshly enriched $CD44^{+/-}$ cells will be suspended in DMEM F12 serum-free medium (Life Technology, Milan, Italy), and the stained cells will be analysed and counted under a fluorescence microscope. In this study, we define all of the $CD44^+$ cells as CSCs.

Data collection and monitoring

Trained researchers will collect data and record it in the Case Report Form (CRF). Any amendments to the study protocol will be made in advance and approved by the Ethics Committee of our hospital. To ensure the reliability and confidentiality of the study data, all data will be stored in a separate storage room. Access to the database will be restricted to the researchers on this study team. Participants' information will not be made publicly available or shared without written permission from the participants. The data collection and time course are listed in table 1.

Outcomes

Primary endpoints

The primary endpoints of this study are PFS and OS, and reflect the efficacy of both treatment options and are defined as CSCs in peripheral blood and predictive biomarkers (cytokines secreted by NK cells and activated markers of NK cells) that indicate patients who achieve an effective response.

Secondary endpoint

The secondary endpoint is safety (number and severity of adverse events). It focusses on toxicity of both treatment options. The safety profile will be reported using the NCI-CTC version 4.0 classification. We will provide the number and percentage of patients experiencing various adverse events, indicating the severity and their association with the treatment. Furthermore, we will report all cases in which the treatment was suspended due to an adverse event, as well as all deaths resulting from toxicity.

Safety and adverse event monitoring

Adverse events will be evaluated at the 6th, 12th and 24th week by conducting non-directive questioning of participants. Participants will have the option to voluntarily report any adverse events. Additionally, physical tests, laboratory tests and other assessments may be conducted to identify adverse events. All details of adverse events will be documented in the CRF. The assessment of adverse events will be based on the NCI-CTC version 4.0 criteria, and the proportion of patients experiencing toxicity will be determined based on the severity level.

Statistical analysis

To evaluate the survival ratio, we will calculate the median survival time for both groups. We will employ a reverse Kaplan-Meier approach to assess the survival curve and a log-rank test to determine any differences between the two groups. Additionally, we will conduct Cox regression analysis to examine the potential factors that may impact treatment outcomes. The stepwise Cox regression or Lasso-Cox regression method will be applied to identify relevant factors, and a Cox regression equation will be established to demonstrate the influence of these factors through the regression coefficients.

All statistical evaluations will be carried out using SPSS software (V.22.0). A significance level of $p<0.05$ will be considered statistically significant for all analyses.

Patient and public involvement

Patients/the public will be involved in research design, recruitment and conduct of this study and the development of the informed consent document. All enrolled patients will have the opportunity to receive updates on the study findings on publication.

ETHICAL CONSIDERATIONS AND INFORMED CONSENT

This trial will be conducted in accordance with the Declaration of Helsinki Guidelines for Clinical Research. The study has been reviewed by the Clinical Research Ethics Committee of our hospital (No. 2023LLSC006). Prior to randomisation, all patients will be required to sign informed consent forms, and they have the option to withdraw from the trial at any time. The participants in this study may directly benefit from their participation.

The study aims to provide essential information about the efficacy and safety of NK cells injection combined with XELOX chemotherapy in patients with postoperative stage II–III CRC. Additionally, the study may identify predictive biomarkers for treatment mechanism prediction. The results of this clinical trial will be reported in the future. All documents related to the trial, including CRF, are recorded and classified using subject identification codes rather than subject names.

Table 1 Time course for data collection and follow-up

Enrolment and allocation	Enrolment and allocation		Treatment period (weeks)								Follow-up period (months)								
Time point	**Screening (baseline)**	**Allocation (baseline)**	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**30**	**36**
Demographics	×																		
Vital signs	×																		
Medical history	×																		
Inclusion/exclusion assessment	×																		
Informed consent		×																	
Routine blood	×		×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Liver function	×		×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Kidney function	×		×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Tumour markers	×		×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Electrocardiograph	×		×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Chest CT scan											×		×		×		×	×	×
Abdominal contrast-enhanced CT											×		×		×		×	×	×
Coloscopy											×		×		×		×	×	×
Allocation		×																	
Treatment		×	×	×	×	×	×	×	×	×									
NK cells markers (NCRs, CD56, CD16)			×	×	×	×	×	×	×	×	×		×		×		×	×	×
Cytokines (IFN-γ and TNF-α)			×	×	×	×	×	×	×	×	×		×		×		×	×	×
CSCs							×				×		×		×		×	×	×
Adverse events		×	×	×	×	×	×	×	×	×									

CSCs, cancer stem cells; IFN-γ, interferon-gamma; TNF-α, tumour necrosis factor-alpha.

Dissemination policy

The results of this study will be published in peer-reviewed journals regardless of whether the results are negative, positive or inconclusive. Efficacy and safety results will be published as soon as the data becomes available during the study period. Furthermore, the study has been registered at www.chictr.org.cn, and the results will be updated on this website as the study progresses and published after its completion.

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Contributors SY conceived and designed the study and drafted the manuscript. XY drafted the statistical plan and calculated the sample size. SG revised the manuscript for important intellectual content, read and approved the final manuscript.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not applicable.

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