

Disappearance of bone metastases in chemotherapy-resistant gastric cancer treated with antigen peptide-pulsed dendritic cell-activated cytotoxic T lymphocyte immunotherapy: A case report

JUAN DU, JIA WEI, YANG YANG, SHU SU, JIE SHAO, FANGJUN CHEN,
FANYAN MENG, ZHENGYUN ZOU and BAORUI LIU

The Comprehensive Cancer Center of Drum Tower Hospital, Medical School of Nanjing University and
Clinical Cancer Institute of Nanjing University, Nanjing, Jiangsu 210008, P.R. China

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Abstract. The adoptive transfer of cytotoxic T lymphocytes (CTLs) stimulated by specific tumor antigen peptide-pulsed dendritic cells (DCs) is one of the most promising immunotherapeutic strategies currently available for patients with gastric cancer (GC). The present case report describes a patient with chemotherapy-resistant stage IV GC with multiple bone metastases, who had been treated with antigen peptide-pulsed DC-CTLs. DCs and CTLs were transfused into the patient subcutaneously and intravenously with simultaneous oral administration of low-dose cyclophosphamide. Following 3 cycles of combination therapy, marked remission regarding the number of metastatic bone lesions was achieved, confirmed by the use of enhanced computerized tomography, computerized tomography and magnetic resonance imaging. After 1 year, 8 cycles of adoptive immunotherapy were administered, and a further decrease in the number of metastatic bone lesions was observed in addition to a marked improvement in the patient's quality of life. Therefore, personalized antigen peptide-pulsed DC-CTLs combined with oral administration of low-dose cyclophosphamide may serve as a promising anticancer therapy to eradicate tumor cells, and therefore this approach is recommended for future cases of a similar nature.

Introduction

Gastric cancer (GC) is the second most frequent cause of cancer-associated mortality in China (1). Due to the majority of patients do not have specific clinical symptoms and most patients are in late stage at first presentation, its local infiltration and remote metastasis lead to difficult radical operation (2). Despite improvements in the surgical treatment of gastric adenocarcinoma, the recurrence rate remains high particularly in patients with advanced stage disease and surgery alone is an insufficient treatment modality (3).

Chemotherapy has beneficial effects on survival rates in patients with advanced-stage GC; however, overall survival times remain at ~1 year after diagnosis (2). Toxic side effects of chemotherapy have motivated the research and development of less harmful alternatives, including targeted therapy and immunotherapy. Novel approaches using inhibition of human epidermal growth factor receptor 2 (HER2) have demonstrated significant improvements in progression-free and overall survival, compared with chemotherapy alone, in first-line treatment of patients with an overexpression of HER2 (3). In addition, second-line treatment with the vascular endothelial growth factor receptor-inhibitor ramucirumab demonstrated significant benefits in terms of overall survival, compared with best supportive care, in randomized studies (4).

Adoptive cellular immunotherapy (ACI) has been demonstrated to be a promising cancer therapeutic and has achieved partial success in numerous countries over the last two decades (5). In recent years, the importance of personal antigen-based antitumor immunotherapy has grown rapidly. The adoptive transfer of *in vitro* generated tumor antigen-specific cytotoxic T lymphocytes (CTL) provides a promising approach to the immunotherapy of cancer (6). Published randomized clinical trials have established its safety and efficacy in different types of cancer (6,7). However, to the best of our knowledge, the disappearance of multiple bone metastases in chemotherapy-resistant GC with this type of treatment has not been reported.

Correspondence to: Professor Baorui Liu, The Comprehensive Cancer Center of Drum Tower Hospital, Medical School of Nanjing University and Clinical Cancer Institute of Nanjing University, 321 Zhong Shan Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: dujuanglyy@163.com

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The present case report describes a patient with chemotherapy-resistant GC with multiple bone metastases received antigen peptide-pulsed dendritic cell (DC)-cytotoxic T lymphocyte (CTL) therapy combined with oral administration of low-dose cyclophosphamide, and achieved marked remission lasting >1 year.

Case report

Patient history. Written informed consent was obtained from the patient for publication of the present case report and any accompanying results. Ethical approval was obtained from the ethics committee of Drum Tower Hospital, Nanjing, China. A 62 year-old female was diagnosed as recurrent GC with multiple bone metastases at Drum Tower Hospital (Nanjing, China) in September 2013. She had followed an initial surgical removal of a stage IIIC poorly differentiated adenocarcinoma 5 years previously. The patient presented with whole body arthralgia and bone pain. Fig. 1 presents the Radionuclide bone imaging of the patient at four stages of the therapy (Fig. 1A, prior to chemotherapy; Fig. 1B, after chemotherapy and prior to immunotherapy; Fig. 1C, after 3 cycles of immunotherapy; and Fig. 1D, after 8 cycles of immunotherapy). Fig. 2 presents the enhanced computed tomography (CT) of the patient at three stages of the therapy (Fig. 2A, prior to immunotherapy; Fig. 2B, after 3 cycles of immunotherapy; and Fig. 2C, after 8 cycles of immunotherapy). Fig. 3 presents the magnetic resonance imaging of the patient at two stages of the therapy (Fig. 3A, prior to immunotherapy, and Fig. 3B, after 8 cycles of immunotherapy). Radionuclide bone imaging (Fig. 1A), enhanced computed tomography (CT) (Fig. 2A) and magnetic resonance imaging (MRI) (Fig. 3A) revealed multiple bone metastases. The patient underwent 6 cycles of combined chemotherapy with S-1 and docetaxel; however, this caused an increase in metastatic lesions and bone pain. Further radiological investigation demonstrated disease progression and resistance to chemotherapy (Fig. 1B). In addition, the patient experienced severe adverse reactions including neurotoxicity and nausea [World Health Organization (WHO) grade III] due to the chemotherapy regime, and was therefore unable to tolerate the cytotoxic side effects. In June 2014, the patient was recommended to undergo adoptive immunotherapy with personalized antigen peptide-pulsed DC-CTLs.

Selection of targeted antigen peptides. The patient's human leukocyte antigen (HLA)-typed was performed by sequence-specific primer amplification (PCR-SSP) using One Lambda DNA generic typing trays (One Lambda; cat. no. Lot 006; Thermo Fisher Scientific, Inc.) pre-coated with primers specifically designed to detect HLA polymorphism. According to the manufacturer's protocol. The PCR reaction system was 10 μ l in total, and contained 100 ng DNA template. The amplification conditions were as follows (8): For first cycle of denaturation at 96°C for 130 sec and renaturation at 63°C for 10 sec, followed by 9 cycle jumps between 96°C for 10 sec and 63°C for 1 min. Further 20 cycles of denaturation at 96°C for 10 sec, annealing at 59°C for 50 sec, and polymerization at 72°C for 30 sec. The PCR amplified products were examined by agarose gel electrophoresis, and the results were observed under a UV lamp and photographed. According to

the resulting fragment size, the genotype was determined by contrast with the Micro SSP™ HLA class I DNA typing interpretation card (One Lambda; Thermo Fisher Scientific, Inc.) and the One Lambda DNA/LMT software, version 3.98 (One Lambda; Thermo Fisher Scientific, Inc.). The result was positive for HLA-A*2402. Patient tumor samples (5 μ m slices of paraffin-embedded stomach cancer) were obtained and underwent immunohistochemical staining analysis. IHC staining was performed on paraffin-embedded histological sections (thickness, 5 μ m) that were fixed in 10% buffered formalin for 24–48 h, using a polymer peroxidase method (9) (Envision+HRP; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). The operation was performed using the Envision™ kit (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Briefly, following deparaffinization with xylene and anhydrous ethanol (95, 85 and 80% for 4 min each), the tissue sections were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity, followed by incubation with the primary antibodies [carcinoembryonic antigen (CEA) primary antibody, rabbit anti-human polyclonal antibody; cat. no. ZA-0063; 1:100; and vascular endothelial growth factor receptor 2 (VEGFR-2) primary antibody, rabbit anti-human polyclonal antibody; cat. no. ZA-0287; 1:100; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China] in a humidified chamber at 4°C overnight. Slices were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (cat. no. SM801; ENvision + kit; Dako; Agilent Technologies, Inc) for 30 min at room temperature. The immunoreactivity was visualized using a 3,3'-diaminobenzidine for 5 min and hematoxylin stain for 2 min at room temperature. Expression of these proteins was evaluated using optical microscopy (BX43; Olympus Corporation, Tokyo, Japan) as positive when the nucleus of the cancerous tissue was stained. The staining of each specimen was evaluated at magnification, x40 or x400. The rate of positive-stained cancer cells was evaluated in three randomly selected areas (size, 200x200 μ m) from the tumor specimens. When the average positive tumor rate was >10%, the tumor was defined as being positively stained. Positive controls (tissues known to be positive for antigen expression) and negative controls (replaced primary antibody with normal rabbit (rat) serum and the result was negative) were included in each run. Results demonstrated overexpression of CEA and VEGFR-2. CEA-652 peptide (TYACFVSNL; cat. no. 04010007767; GenScript, Co., Ltd., Nanjing, China) (10) and VEGFR-2-169 peptide (RFVPDGNRI; cat. no. 04010013003; GenScript, Co., Ltd.) (11) were used for DC loading according to protocols previously described (12).

Ex vivo expansion of DCs and CTLs obtained from peripheral blood. Peripheral blood mononuclear cells (PBMCs) were collected using the peripheral blood mononuclear cell collection program COBE Spectra™ MNC program (Terumo BCT Co., Ltd., Lakewood, CO, USA) and transported to the laboratory under cold conditions (4°C). *In vitro* cell processing and expansion were performed in a good manufacturing practices-compliant laboratory. DCs and CTLs were obtained from blood mononuclear cells as described previously (12,13). DCs were loaded with HLA-A*2402-binding peptides derived

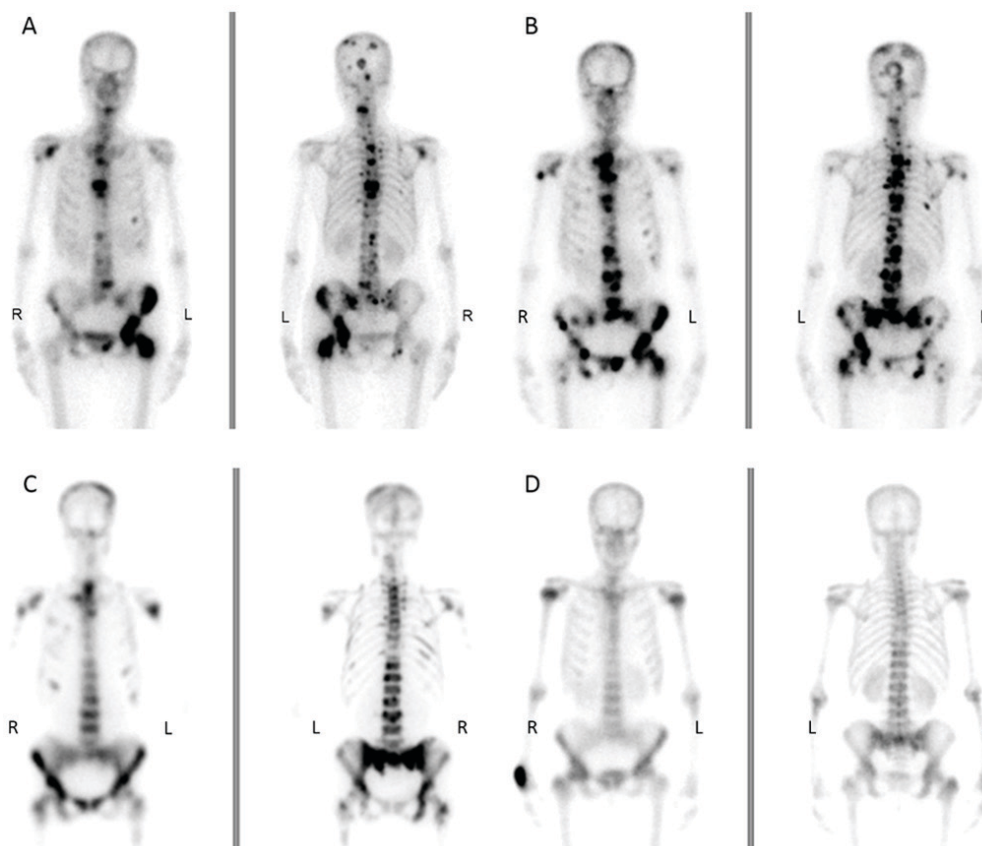


Figure 1. Radionuclide bone imaging scans demonstrating that the bone metastases was markedly increased following 6 cycles of chemotherapy. However, the focus was decreased following 3 cycles of antigen peptide-pulsed dendritic cell-cytotoxic T lymphocyte immunotherapy and was markedly decreased following 8 cycles of immunotherapy. (A) Prior to chemotherapy, (B) following chemotherapy and prior to immunotherapy, (C) following 3 cycles of immunotherapy, (D) following 8 cycles of immunotherapy. R, right; L, left.

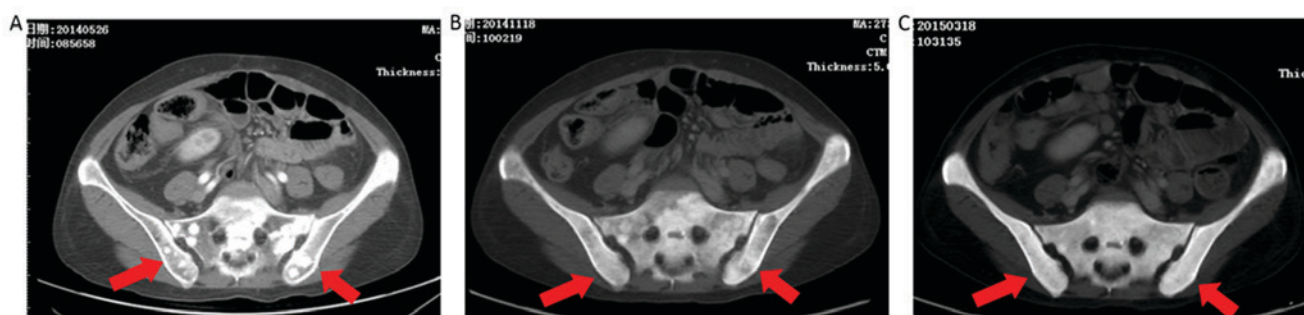


Figure 2. Computed tomography scan demonstrating multiple patchy high-density shadows in the pelvis. Shadows were decreased following antigen peptide-pulsed dendritic cell-cytotoxic T lymphocyte immunotherapy. Red arrows signify the high-density focus of bone metastases. (A) Prior to immunotherapy, (B) following 3 cycles of immunotherapy, (C) following 8 cycles of immunotherapy.

from CEA and VEGFR-2, and CTLs were stimulated with mature DCs and cultured in complete medium containing 10% human AB serum (Gibco; Thermo Fisher Scientific, Inc.) and 1-2% T cell growth factor (Gibco; Thermo Fisher Scientific, Inc.). Cell counts were estimated using the trypan blue dye-exclusion method (14). The average cell count of PBMCs prior to each expansion was 2.7×10^8 , and, after 2 weeks of *in vitro* expansion, the mean cell count of expanded lymphocytes for each injection was 3.9×10^{10} . The mean frequency of activated CTLs [cluster of differentiation $CD3^+/CD8^+$] was determined using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). FACS Ariar cell sorter (BD Biosciences) was used to

perform fluorescent expression analysis. Cells were harvested and stained with mouse anti-human antibody labeled by direct fluorescence antibody for 30 min in 4°C , in darkness as follows: CD3-FITC (HIT3a; cat. no. 561806; BD Biosciences), CD8C-PE (HIT8a; cat. no. 561949; BD Biosciences). The fluorescence antibody was diluted 1:40 and PBS containing 1% BSA were used as the dilution and washing buffer. Cells were washed two times by centrifuging at the speed of $250 \times g$ for 5 min at 4°C . Data were analyzed using FlowJo software 7.6.1 (Tree Star, Inc., Ashland, OR, USA) following gating for $CD3^+$ T cells. Results revealed an initial average frequency of 40%; however, the final culture revealed an

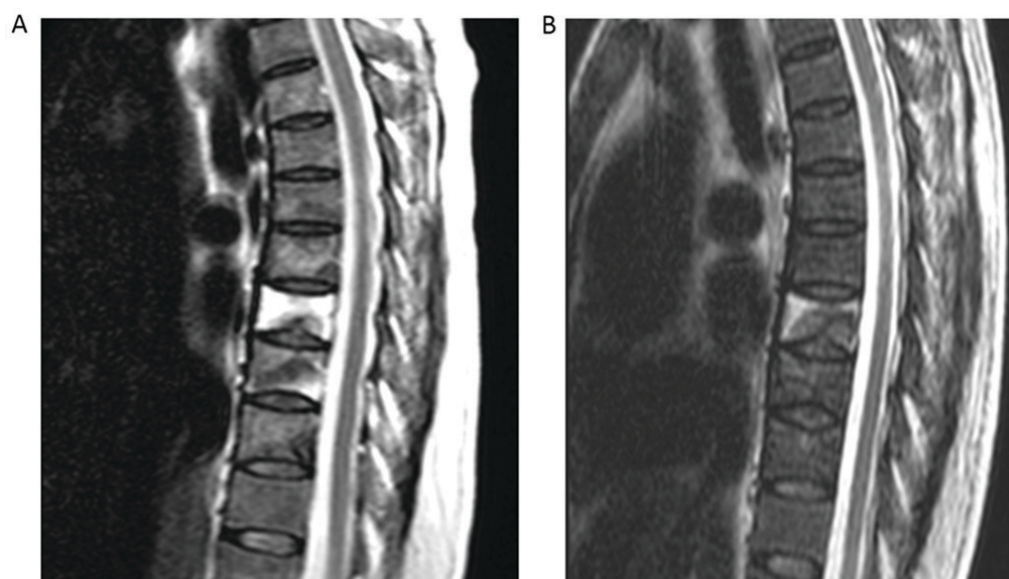


Figure 3. Magnetic resonance imaging scans demonstrating that the high signal areas of T8 and T9 vertebral metastases were markedly decreased following 8 cycles of immunotherapy. (A) Prior to immunotherapy, (B) following 8 cycles of immunotherapy. T, thoracic.

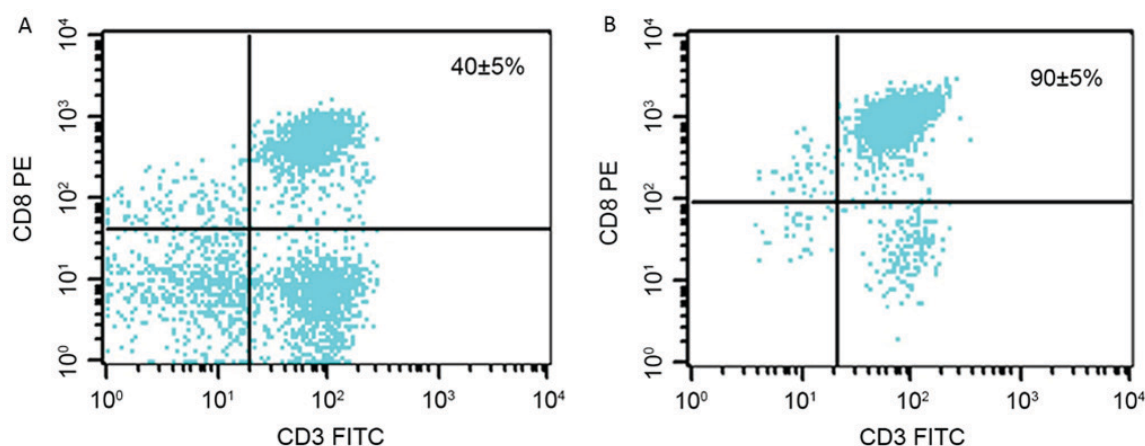


Figure 4. Comparison of the frequency of activated CTL ($CD3^+/CD8^+$) cells prior to and following cell expansion as evaluated using flow cytometry. (A) At baseline, (B) following *ex vivo* expansion. CTL, cytotoxic T lymphocyte; CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

average activation of 90% (Fig. 4). Each CTL infusion was accompanied by a 5-day course of low-dose interleukin-2 (IL-2; 4×10^6 U daily). Prior to cell transplantation, cells were tested for endotoxin levels using a Limulus Amebocyte Lysate kit (Charles River Laboratories, Ltd., Mumbai, India) to ensure that products were aseptic. Interferon- γ enzyme-linked immunospot (ELISPOT) assay was conducted using PBMCs periodically obtained from the patient prior to and following immunotherapy in order to assess cellular immune responses to CEA and VEGFR-2. IFN- γ ELISPOT kit (Shenzhen Dakewei Biotechnology Co. Ltd, Anshan, China) was used to determine the frequency of cytokine-expressing T cells following overnight activation with peptides at 37°C. The kit was used according to manufacturer's protocol. Briefly, T cells (105 cells per well) and peptides (50 mg/ml) were added to duplicate wells and DCs were added at a ratio of 1:10 (DC:T cells) for 18-20 h at 37°C. The plates were washed prior to the addition of the diluted detection antibody (1:100 dilution) and then incubated for 1 h at 37°C, followed by incubation with

streptavidin-alkaline phosphatase-conjugated antibody (1:100 dilution) was added and incubated at 37°C for a further 1 h. Aminoethyl carbazole solution mix was then added to each well, and the plates were left in the dark for 15-25 min at room temperature followed by deionized water being added to stop development. Plates were scanned using an Elispot CTL Reader (Cell Technology Inc., Columbia, MD) and the results were analyzed using ImmunoSpot 5.0.41 Professional (Cell Technology Inc., Cleveland, OH, USA). A paired Student's t-test was applied using Graphpad Prism 5 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Treatment plan. The patient received 8 cycles of antigen peptide-pulsed DC-CTLs with an oral administration of low-dose of cyclophosphamide (50 mg daily) from June 2014 to May 2015. Each cycle included the administration of two DC vaccines ($1.2 \sim 1.6 \times 10^7$ in 1 ml 0.9% sodium chloride injection) and four infusions of CTLs ($0.8 \sim 1.4 \times 10^{10}$ in 100 ml

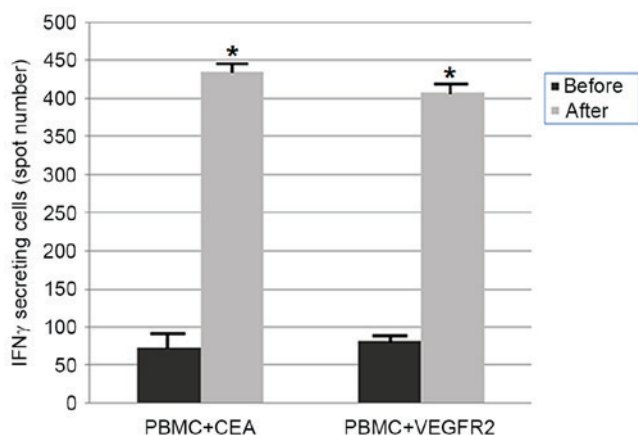


Figure 5. IFN- γ ELISPOT analyses of the patient's PBMC response to CEA and VEGFR-2 prior to and following immunotherapy. The average number of IFN- γ spots observed per 10^5 PBMCs plated was recorded by ELISPOT analysis. * $P < 0.05$ vs. prior to immunotherapy. IFN, interferon; PBMC, peripheral blood mononuclear cell; CEA, carcinoembryonic antigen; VEGFR-2, vascular endothelial growth factor receptor 2; ELISPOT, enzyme-linked immunospot.

0.9% sodium chloride injection). The DCs and CTLs were administered intradermally and intravenously, respectively. Throughout the course of treatment, the progression of the metastatic bone lesions was monitored using CT, MRI and ECT. Adverse clinical effects following large-dosage lymphocyte transfusions were mild and consisted of chills and fever (WHO grade I) and typically occurred within 6-8 h of CTL infusion, with recovery following symptomatic treatment with acetaminophen (0.3-0.6 g each time, orally, three times a day).

Results

Evaluation of the immune response. Interferon- γ ELISPOT assays were conducted using PBMCs periodically obtained from the patient prior to and following treatment in order to assess cellular immune responses. Positive CTL responses specific to the vaccinated peptides were determined as previously described (10). Interferon- γ secretion was significantly higher on CTL by the stimulation of HLA-A*2402-binding peptides CEA and VEGFR-2 following 4 cycles of immunotherapy compared with control T cells prior to immunotherapy ($P < 0.05$) (Fig. 5).

Imaging evaluation of clinical response. Following 3 cycles of treatment, CT scan demonstrated a decrease in multiple patchy high-density shadows located in the pelvis (Fig. 2B). The radionuclide bone-imaging scan indicated a marked decrease in the number of multiple bone metastases (Fig. 1C). Following completion of 8 cycles of autologous immune enhancement therapy, the number of multiple bone metastases was markedly decreased, this was further confirmed in June 2015 using CT imaging (Fig. 2C), a radionuclide bone imaging scan (Fig. 1D) and an MRI scan (Fig. 3B). Furthermore, the patient's weight had increased, appetite and quality of life were improved and the arthralgia and bone pain were also relieved. Overall, the patient was doing well at the time of writing.

Discussion

The present case was the first report of the disappearance of almost all bone metastases following antigen peptide-pulsed DC-CTLs immunotherapy in chemotherapy-resistant gastric cancer. We also investigated the safety and efficacy of the *ex vivo* activated, expanded and adoptively transferred antigen peptide-pulsed DC-CTLs obtained from the peripheral blood of a stage IV chemotherapy-resistant patient with GC and multiple bone metastases, whose bone metastases was markedly decreased following therapy and without serious adverse reactions.

As an immune-based approach, adoptive therapy has become an increasingly attractive modality for cancer therapy due to collectively demonstrating a decreased risk of cross-resistance compared with conventional therapies, high specificity and long-term immune protection (15). Recent success of adoptive T cell therapy using *ex vivo* expanded autologous tumor-reactive T cells has increased optimism that this modality may form a specific therapy for patients with advanced-stage disease, including those who are refractory to standard therapies (16,17). Previously, autologous immune cells intravenously administered to patients with GC have resulted in improved survival rates compared with controls, as reported by Zhang *et al* (18).

CEA and VEGFR-2 are the most commonly expressed antigens in GC. CEA epitopes and VEGFR-2 epitopes presented in the HLA-A*0201 and HLA-A*2402 context are frequently recognized by T cells (10,11). As a result, the majority of recent adoptive cell therapy protocols have targeted these antigens and reported encouraging immunological and clinical responses (10,11). Over the course of an adoptive cell transfer, monitoring antigen-specific T cell expansion in patient blood is crucial for predicting the clinical efficacy of such an immunological approach. The patient was administered with *ex vivo* generated CEA- and VEGFR-2-specific CTLs. Following 4 cycles of adoptive transfer, the ELISPOT results demonstrated that the number of antigen-specific T cells were markedly increased in the patient's blood.

The clinical efficacy of current tumor immunotherapy methods is not satisfactory as immunosuppressive mechanisms [including regulatory T cells (Treg) and myeloid-derived suppressor cells] are predominant in patients with cancer at an advanced stage (19-21). A previous study demonstrated that cyclophosphamide exerted variable effects on depleting suppressive Tregs, which resulted in enhanced T cell reactivity (22). Research also demonstrated that cyclophosphamide at a low dose selectively depleted Tregs, whereas at a high dose, cyclophosphamide led to a loss in specificity of Treg depletion in patients with cancer (23). Using this strategy, marked clinical benefits have been obtained, such as a significant delay in tumor progression. Numerous proof-of-concept clinical trials in patients with cancer indicated that the efficacy of adoptive cellular therapy may be enhanced using chemotherapy (24-26). In the present case report, oral administration of low-dose cyclophosphamide accompanied by immune cell transfusions resulted in the almost complete disappearance of all multiple bone metastases in chemotherapy-resistant GC, which lasted for over one year. Therefore, optimizing the combination of adoptive cellular therapy and other

immune-based or conventional approaches may herald a new generation of research and clinical opportunities for cancer immunotherapy.

In conclusion, specific antigen peptide-pulsed DC-CTLs combined with low-dose cyclophosphamide administered to the patient with stage IV chemotherapy-resistant GC, demonstrated its safety by not exhibiting any severe adverse reactions. There was also a marked decrease in bone metastatic lesions, improved quality of life and a prolonged duration of progression-free survival over one year. This is also in agreement with previous studies (27-29) on cell-based immunotherapies. Therefore, it is recommended that the adoptive transfer of personalized antigen peptide-pulsed DC-CTLs may serve as a promising specific immunotherapy strategy for patients with malignant disease, and may be considered in combination with oral administration of low-dose cyclophosphamide or other modalities of treatment in cases of a similar nature.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JD and BL undertook study conception and design. JD, JW, YY, SS, JS, FC, FM and BL were responsible for the collection and assembly of data: Data analysis and interpretation were the responsibilities of JD, SS, JS, FC, FM, ZZ and BL. JD wrote the manuscript. Final approval of manuscript was given by JD, JW, YY, SS, JS, FC, FM, ZZ and BL.

Ethics approval and consent to participate

Ethical approval was obtained from the ethics committee of Drum Tower Hospital, Nanjing, China. Study participants provided written informed consent.

Consent for publication

Written informed consent was obtained from the patient for publication of the present case report and any accompanying results.

Competing interests

The authors declare that they have no competing interests.

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