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Safety and efficacy study of nasopharyngeal cancer stem cell vaccine

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ABSTRACT

In this trial, nasopharyngeal cancer stem cells (CSCs) were separated and cultured to produce a vaccine; its safety and efficacy were prospectively evaluated in low-, medium-, and high-dose groups. Between April and September 2014, we enrolled 90 patients who met the enrolment criteria, and assigned them to three groups ($n = 30$). Throughout the trial, injection site reaction was the most common reaction (81%), and fever was least common (31%); however, there was no difference among the three groups. When the immune responses pre- and post-vaccination were compared, we found that the CSC-specific and -nonspecific response in the medium- and high-dose groups were both significantly enhanced. This study is the first clinical trial of a nasopharyngeal CSC vaccine and preliminarily proves its safety and efficacy.

1. Introduction

Nasopharyngeal cancer-related mortality is almost invariably due to metastasis [1,2]. Between 25% and 50% of patients diagnosed with nasopharyngeal cancer will eventually develop deadly metastases, often years after surgery or radiotherapy [3,4]. The therapeutic alternatives for metastatic nasopharyngeal cancer are mainly based on the systemic administration of cytotoxic chemotherapeutic agents; the long-term impact on survival, depends greatly on the nature of the metastases and tumor biology [5,6]. Many pilot studies have confirmed the good therapeutic effects and fewer side effects of percutaneous ablation, primarily in recurrent cancer [7,8]. Immunotherapy may have powerful therapeutic effects under low burden load conditions [9,10].

Traditional immunotherapy supplements lymphocyte count and function effectively, but has no tumor specificity, and it is difficult to form a memory immune response for long-term prevention of tumor recurrence [11,12]. Currently, accumulating evidence suggests that a tumor cell subpopulation with distinct stem cell-like properties (the so-called cancer stem cells, CSCs) is responsible for the initiation, invasion, and metastasis of nasopharyngeal cancer [13,14]. In the laboratory, CSCs are identified by the CD44 surface marker and their ability to form new nasopharyngeal cancer colonies through serial transplantations in immunodeficient hosts, reestablishing tumor heterogeneity [15,16].

To assess the feasibility of generating CSC vaccines for clinical use, we harvested peripheral blood and tumor specimens from patients with nasopharyngeal cancer. Lymphocytes were isolated from peripheral blood mononuclear cells (PBMCs); CSCs were isolated from the tumor specimens. CSC-specific and -nonspecific immunity induced by cytotoxic T cells and antibodies, and adverse event occurrence were compared before and after the trial.

2. Material and methods

2.1. Ethics

This clinical trial was registered in the US National Institutes of Health (ID: NCT02115958; Ph1/Ph2) and approved by the Ethics Committee of Guangzhou Fuda Cancer Hospital. In accordance with the Declaration of Helsinki, written informed consent was obtained from each participant in Fuda Cancer Hospital.

Abbreviations: CSC, cancer stem cell; PBMC, peripheral blood mononuclear cells; CDC, complement-dependent cytotoxicity; CTL, cytotoxic T lymphocyte; DC, dendritic cell.

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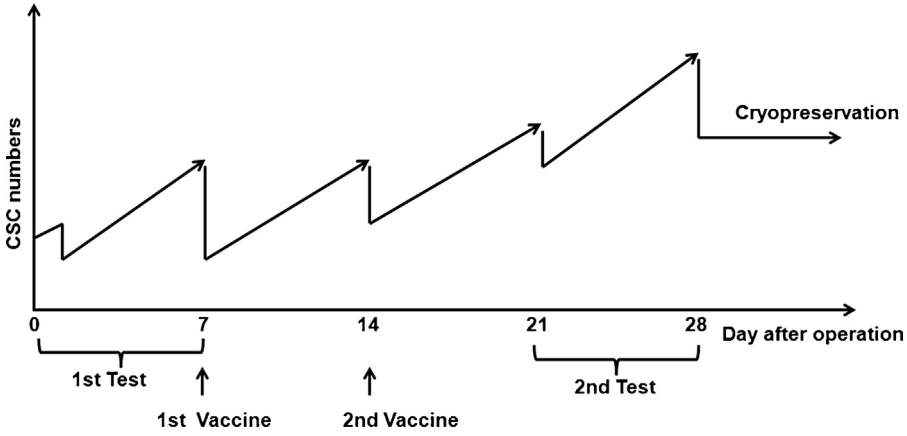

Fig. 1. Vaccination and test schedule, and trend chart of CSC numbers. Broken lines represent the gradual amplification of CSCs and single consumption during vaccination or testing.

2.2. Patient selection

This was a prospective study of the therapeutic effects of a CSC vaccine for nasopharyngeal cancer patients enrolled between April and September 2014. We enrolled 90 patients using the following criteria: (1) had at least one resectable tumor, and other tumors could be well-controlled by cryosurgery, brachytherapy, or chemotherapy, (2) expected survival >3 months, (3) aged 30–75 years, (4) Karnofsky performance status >70, (5) the following parameters were normal: total T cells, 603–2990 μL^{-1}; cytotoxic T cells, 125–1312 μL^{-1}; helper T (Th) cells, 441–2156 μL^{-1}; platelets $\geq 80 \times 10^9\ L^{-1}$; white blood cells $\geq 3 \times 10^9\ L^{-1}$; neutrophils $\geq 2 \times 10^9\ L^{-1}$; hemoglobin ≥90 g/L; time international normalized ratio, 0.8–1.5; adequate hepatic function (bilirubin <20 μM, aminotransferase <60 U/L) and renal function (serum creatinine <130 μM, serum urea <10 mM), (6) absence of level 3 hypertension, severe coronary disease, myelosuppression, respiratory disease, acute or chronic infection, and autoimmune diseases. The contraindications for participation were T cell lymphoma, ongoing organ transplant or within 7 days after systemic chemotherapy. The enrolled patients were allocated to three groups ($n = 30$) with parallel assignments.

2.3. Preparation of CSC vaccine

The CSC vaccine contained three components: nasopharyngeal CSC fragments, Multi-oil Fat Emulsion Injection (C_{6-24}; Fresenius Kabi GmbH, Graz-Puntigam, Austria), and Mannatide Injection (Duokang; Chengdu Lier Pharmaceutical, Chengdu, China). The total vaccine volume was 0.5 mL: 0.3 mL CSC/saline solution (10^5, 3×10^5, and 5×10^5 CSCs for the low-, medium- and high-dose group, respectively), and 0.1 mL each of the remaining components.

The CSC preparation process involved seven steps: (1) solid tumor resection (e.g. primary lesion or metastasis in liver or lung, generally diameter >4 cm), (2) preparation of single-cell suspensions using a human Tumor Dissociation Kit (no. 130-095-929; Miltenyi Biotec, Bergisch Gladbach, Germany), (3) CSC separation using a human CD44 MicroBead Kit (no. 130-095-194; clone MC56; Miltenyi Biotec), (4) CSC amplification and culture using a human StemMACS MSC Expansion Media Kit XF (no. 130-104-182; Miltenyi Biotec), (5) detection of cell purity by phycoerythrin (PE) anti-human CD44s (no. 130-095-180; clone DB105; Miltenyi Biotec), (6) cell counting and reserving at different concentrations, (7) CSC lysis by repeated freezing and thawing five times. All steps were performed in accordance with the corresponding instructions.

2.4. Vaccinations and tests

Based on our previously reported animal experiments [17], the vaccination period involved two subcutaneous injections on both sides of the deltoid muscle, interspersed with a 1-week interval. Before and 1 week after vaccination, ~30 mL peripheral blood was drawn for in vitro testing (Fig. 1).

Peripheral blood (2 mL) was used for detecting CSC-nonspecific immunity by flow cytometry (FACSCanto™ II; BD, Franklin Lakes, NJ, USA). Multitest 6-color TBNK Reagent (no. 644611; BD) was used to detect the number of lymphocyte subsets; Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II (no. 551809; BD) was used to detect cytokine expression levels. All steps were performed in accordance with the corresponding instructions.

Using Ficoll-Hypaque density centrifugation, PBMCs and plasma were harvested from the remaining blood samples, and CSC-specific immunity induced by cytotoxic T cells and antibodies were compared before and after the trial. CSC-specific testing included CSC binding by immune plasma, CDC and CTL tests. Plasma CSC-binding antibodies were detected using fluorescein isothiocyanate anti-human immunoglobulin G (IgG, no. 130-099-229; clone IS11-3B2.2.3; Miltenyi Biotec); we have described the protocols previously [17]. T lymphocytes were separated by human CD3 MicroBeads (no. 130-050-101; Miltenyi Biotec), activated by anti-human CD3/28 (no. 300314/302934, BioLegend, San Diego, CA, USA), and expanded using interleukin (IL)-2 (no. 200-02, PeproTech, Rocky Hill, NJ, USA); DCs were induced and cultured in IL-4 (no. 200-04, PeproTech) and granulocyte-macrophage colony-stimulating factor (no. 300-03, PeproTech); we have described the protocols previously [18].

2.5. Evaluation and statistical analysis

Complications were recorded and classified in accordance with the Common Terminology Criteria of Adverse Events Version 4.02. Radiographic local tumor control was assessed using image-guided tumor ablation criteria [19]. Patient demographics were compared among the three groups before vaccination by two-way analysis of variance (ANOVA); CSC-specific and -nonspecific immunity pre- and post-vaccination were compared by Dunnett's multiple comparison test (one-way ANOVA), detection result data are presented as the mean ± standard deviation; local and systemic

Table 1
Patient demographics.

Patient characteristics before vaccination	Total (n = 90)	Low-dose group (n = 30)	Medium-dose group (n = 30)	High-dose group (n = 30)	P value
Gender (male/female)	40/50	13/17	15/15	12/18	>0.05
Median age (year)	51	52	54	47	>0.05
Differentiated degree of squamous carcinoma					
High	41	16	12	13	>0.05
Moderate	34	10	12	12	>0.05
Poor	15	4	6	5	>0.05
Clinical stage					
II	30	11	7	12	>0.05
III	24	7	9	8	>0.05
IV	36	12	14	10	>0.05
Karnofsky performance status					
80	26	7	9	10	>0.05
90	33	12	10	11	>0.05
100	31	11	11	9	>0.05
Chemotherapy	48	16	17	15	>0.05
Radiotherapy	71	24	23	24	>0.05
Surgery	90	30	30	30	>0.05

Note: Factors of low, middle and high dose group were compared by two-way ANOVA.

adverse events were marked in the nursing records and compared using the chi-square test. All analyses were conducted using GraphPad software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Identification of CSCs in enrolled patients

According to the protocol depicted in Fig. 1, all enrolled patients underwent surgical resection before the CSCs were separated in the lab. Before separation, CSC purity was 1.2 ± 0.2%; after separation, CSC purity increased up to 92 ± 9.5% (Fig. 2). All CSCs separated from the tumor mass were sufficient for culture, vaccination, and testing, and CD44+ cell purity after 2- and 4-week culture was similar to that immediately following separation (figures not shown).

3.2. Clinical data

All pretreatment informations were collected from 90 patients of our hospital, which were from China (31), Indonesia (25) and Mid-East (34), respectively. The data of the three groups were compared, and there was no statistical difference for the patient demographics (Table 1).

3.3. Adverse events

Throughout the trial, all adverse events experienced by the patients were recorded, and included local (mainly injection site reaction or localized edema) and systemic (mainly chills, fatigue, or fever) reactions. Other possible side effects such as blood or bone marrow changes were not detected. All adverse events were Grade 1; after symptomatic treatment, all symptoms were relieved within the day, and did not reappear. Adverse event occurrence was compared using the chi-square test; there was no difference among the three groups (P = 0.875, Fig. 3). Among the patients, the most common reaction was injection site reaction (81%); fever was the least common reaction (31%).

3.4. CSC-nonspecific and -specific immunity

Lymphocyte count and function of the first and second test were compared: the test data of all patients pre-vaccination were merged and compared with the test results obtained post-vaccination (Table 2). From the aspect of lymphocyte count, all subsets in the medium- and high-dose groups were significantly higher after vaccination; from the aspect of lymphocyte function, Th1-type cytokines were higher in the medium- and high-dose groups, while Th2-type cytokine levels were essentially unchanged.

CSC-specific immunity of the first and second test was compared: the test data of all patients pre-vaccination were merged and compared with the test results obtained post-vaccination (Fig. 4). In CSC binding by immune plasma, the proportions of positive

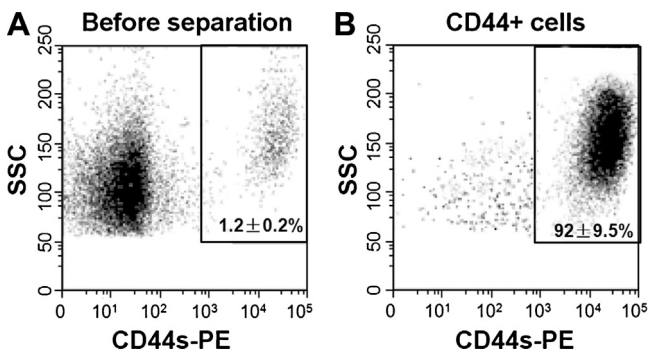

Fig. 2. CSCs in nasopharyngeal cancer mass. (A) CSC purity in tumor cell suspension before separation. (B) CSC purity in separation products obtained using the human CD44 MicroBead Kit.

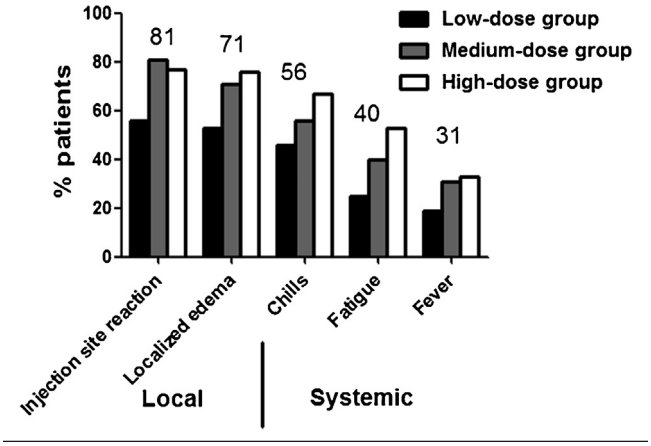

Fig. 3. Local and systemic adverse events. The incidences of each Grade 1 adverse event that occurred in the three groups are depicted in separate columns; figures above the columns indicate the average incidences in all patients.

Table 2
Comparison of number and function of lymphocyte.

Test items of lymphocyte	Testing results			
	Test 1 (n = 90)	Test 2 (low-dose group)	Test 2 (medium-dose group)	Test 2 (high-dose group)
Number (cell/μL)				
Total T cell	1431 ± 66	1509 ± 81*	1606 ± 83***	1829 ± 96***
CD8 + T cell	636 ± 13	698 ± 25	738 ± 36***	782 ± 38***
CD4 + T cell	751 ± 35	813 ± 47*	836 ± 40**	871 ± 36***
NK cell	425 ± 36	536 ± 56	569 ± 67**	637 ± 76***
B cell	326 ± 17	377 ± 15	480 ± 31**	566 ± 38***
Function (pg/mL)				
IL-2	12.5 ± 3.7	14.4 ± 4	14.6 ± 3.8**	24.5 ± 4.9**
TNF-β	4.4 ± 2.8	4.8 ± 3.6	10.5 ± 2***	14.6 ± 2.2***
IFN-γ	5.4 ± 3.1	5.6 ± 2.4	11.5 ± 3***	17.4 ± 3.6***
IL-4	9.5 ± 2.5	9.4 ± 2	9.2 ± 3.5	10.4 ± 3.4
IL-6	12.5 ± 3.2	13.5 ± 3.7	14.3 ± 4	15.4 ± 9.3
IL-10	8.5 ± 2.3	9.4 ± 3.4	8.6 ± 2	9.1 ± 3.1

Note: Every cell subset or cytokine were analyzed by Dunnett's Multiple comparision test (one-way ANOVA). NK cell, natural killer cell; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

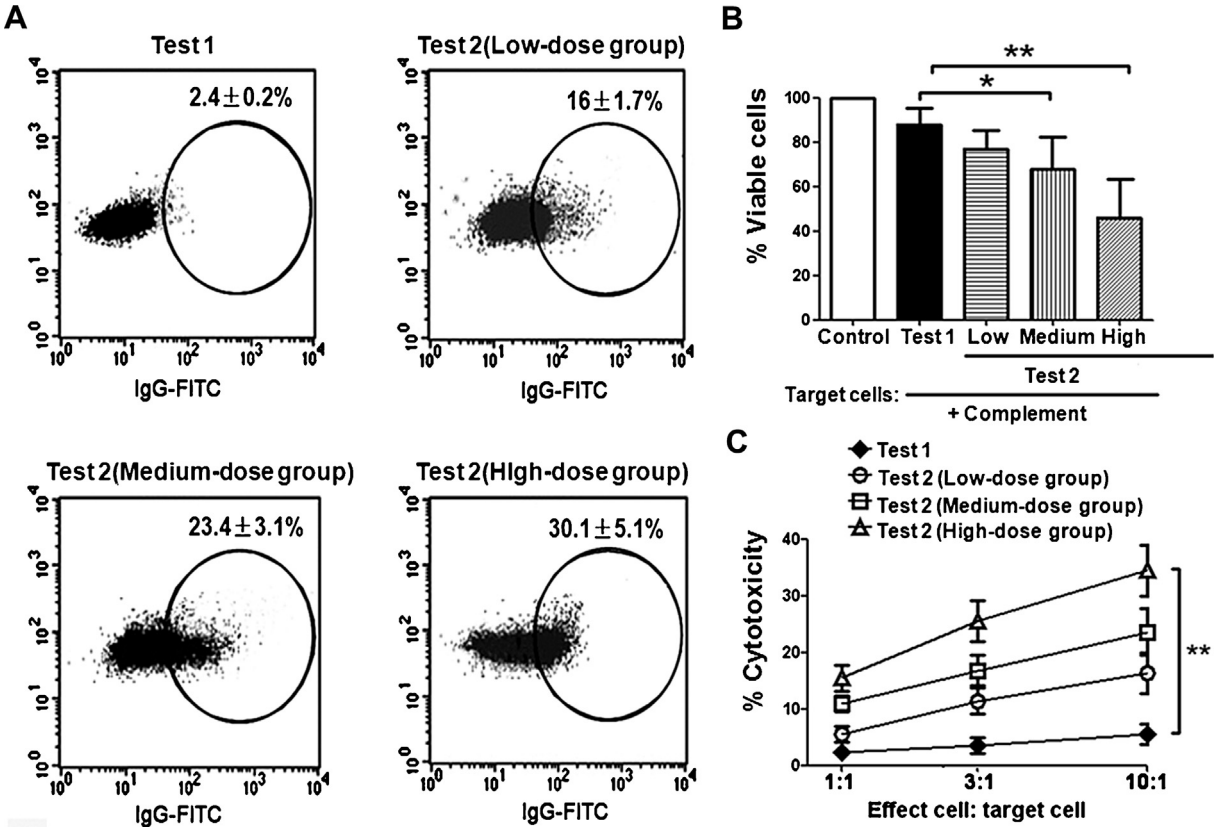

Fig. 4. Comparison of CSC-specific immunity pre- and post-vaccination. (A) Flow cytometry results of CSC-specific antibody levels in immune plasma and IgG levels. (B) CSC targeting by CSC-primed CDC; 10^5 viable CSCs were incubated with immune plasma for 1 h (control group: no immune plasma), then rabbit complement was added and incubated for another 1 h. Viable cells were then counted under a microscope after trypan blue staining. (C) CSC targeting by CSC-primed CTLs; CSC killing was measured by a lactate dehydrogenase release assay. A higher percentage of cytotoxicity indicated more cell lysis. **$P < 0.01$, ***$P < 0.001$.

staining were all significantly higher ($P < 0.001$, Fig. 4A) after vaccination; in CSC destruction by immune plasma, the percentage of viable cells after complement-dependent cytotoxicity (CDC) (83 ± 5% in test 1) was significantly decreased in both the medium-dose (57 ± 12%, $P < 0.01$) and high-dose groups (32 ± 14%, $P < 0.001$) (Fig. 4B). In CSC destruction by cytotoxic T lymphocyte (CTL), the cytotoxicity effects at three effect/target proportions were all significantly increased in the medium- ($P < 0.01$) and high-dose groups ($P < 0.001$, Fig. 4C).

4. Discussion

The subject of some controversy, CSCs are considered capable of symmetric or asymmetric self-renewal, resistance to standard chemotherapies or radiotherapies, less differentiated, and tumorigenic; the last is evinced by the ability of low numbers of CSCs to establish tumors in immunodeficient mice [20,21]. As the failure of current therapies to control cancer can be attributed to their inability to eliminate CSCs, it is critical to develop strategies

that eliminate these stem cell-like tumor cells. Recent data suggest that immune-based approaches may be particularly attractive prospects for targeting CSCs: one strategy is to target CSCs via monoclonal antibodies, such as CD47 [22] and CD123 [23], which have been shown to eradicate leukemia stem cells in preclinical models. Another approach involves harnessing cellular immune responses, such as anti-tumor T cells [24,25] or anti-tumor dendritic cells (DCs) [17,26], which have also produced good effects in animal models. Currently, commercial CSC separation and amplification kits are widely used, offering technical guarantees for the clinical application of this vaccine. This study represents the first trial of a nasopharyngeal CSC vaccine in clinical use, and is a step forward in the exploration of cellular immune responses targeting CSCs.

Fortunately, no blood or bone marrow changes were detected in any patient; local or systemic side effects that appeared were alleviated within the day. From the perspective of efficacy, CSC-specific and -nonspecific lymphocytes were both significantly increased; CSC-specific and -nonspecific immunity were both continually enhanced as the number of CSCs in the vaccine increased, and severe adverse events were not observed.

At least four factors guarantee the safety and efficacy of the vaccine: (1) all patients underwent cytoreductive surgery, radiotherapy, or cryosurgery to reduce the tumor burden, and then received the vaccination. The organ-like structural environment of tumors is very hostile toward immune cells, and can result in local immune tolerance [27]; a low tumor burden provides the necessary conditions for the in vivo survival and amplification of specific lymphocytes. (2) DC activation plays a central role in nasopharyngeal cancer, where the level of tumor-specific lymphocytes decreases prior to tumor recurrence and increases again with immunization [28]. Our experience with animal experiments established the foundations of how and when to vaccinate [17]; although the drawing of extra blood for DC preparation is difficult, the addition of adjuvants (Mannatide Injection) compensated for this drawback and produced a good immune effect. (3) Ongoing CSC cultures ensured antigen activity during vaccination and detection, which provided a further guarantee of the effects of vaccination and detection. (4) Given the individual differences of allogeneic CSCs, individualized CSC vaccines for each patient guarantee a CSC-specific response and accurate activation of the T-cell repertoire.

In this study, immune sera from CSC-vaccinated hosts contained high levels of IgG which bound to CSCs, resulting in CSC lysis in the presence of complement. CTLs generated from peripheral blood mononuclear cells harvested from CSC-vaccinated hosts were capable of killing CSCs in vitro. Enriched CSCs were immunogenic and more effective as an antigen source than unselected tumor cells in inducing protective antitumor immunity [17]. Mechanistic investigations established that CSC-primed antibodies and T cells were capable of selective targeting CSCs and conferring antitumor immunity. Together, these proof-of-concept results provide a rationale for a new type of cancer immunotherapy based on the development of CSC vaccines that can specifically target CSCs.

Altogether, this study first demonstrated the safety and efficacy of the nasopharyngeal CSC vaccine in a clinical trial, but the vaccine requires further refining. At the cellular level, CSCs might modulate the immune responses via several mechanisms. For example, CSCs may selectively not express tumor-associated antigens associated with differentiation and may therefore be resistant to immune-mediated rejection or evade host immunosurveillance through the absence or downregulation of major histocompatibility complex class I molecules [29]. Therefore, the mechanism by which the nasopharyngeal CSC vaccine strengthens the specific and nonspecific response requires further investigation. From the viewpoint of a long-term curative effect, the benefit of this vaccine on progression-free survival and overall survival also requires further research.

Conflict of interest

The authors declare that there are no conflicts of interest.

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