Regular Article

The Safety and Immunological Effects of rAd5-EBV-LMP2 Vaccine in Nasopharyngeal Carcinoma Patients: A Phase I Clinical Trial and Two-Year Follow-Up

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Epstein–Barr virus (EBV)-encoded latent membrane protein 2 (LMP2) promotes nasopharyngeal carcinoma (NPC) progression. Previously, we reported that the dendritic cells (DCs) transfected with EBV-LMP2 recombinant serotype 5 adenoviruses (rAd5) induced anti-tumor effect by eliciting cytotoxic T lymphocytes (CTLs)-mediated immune response in vitro and the adenoviral vaccine of EBV-LMP2 (rAd5-EBV-LMP2) stimulated antigen-specific cellular immunity in mice. However, the safety and immunological effect of rAd5-EBV-LMP2 vaccine in human still remained unknown. Here we conducted a single-center, non-randomized, open-label, single-arm phase I clinical trial to clarify this unsolved issue. A total of 24 patients with regional advanced NPC were sequentially enrolled into three dose level groups (2 × 10⁹, 2 × 10¹⁰, 2 × 10¹¹ vp). The rAd5-EBV-LMP2 vaccines were intramuscularly injected for four times within 28 d (D₁, D₇, D₁₄, D₂₈). Blood samples were harvested immediately before every vaccination, one week and one month after the last vaccination (D₂, D₃, D₄, D₁₅, D₂₉, D₃₀). All the vaccine inoculation-related toxicities presented as grade I/II adverse events. The most frequent systemic adverse reactions were fatigue (33.0%, 8/24), myalgia (29.2%, 7/24) and cough (29.2%, 7/24), while the most common regional adverse reaction was tenderness in the inoculation site (54.2%, 13/24). In addition, proportion of CD³⁺CD⁸⁺ cells in peripheral blood was significantly increased in the high dose group (2 × 10¹¹ vp). The rAd5-EBV-LMP2 vaccine was generally well-tolerated and the high dose (2 × 10¹¹ vp) is recommended to be adopted in phase II studies. The long-term outcome of rAd5-EBV-LMP2 vaccine inoculation is required to be determined in following placebo-controlled trials.

Key words latent membrane protein 2; cancer immunotherapy; nasopharyngeal carcinoma; Epstein–Barr virus

Nasopharyngeal carcinoma (NPC) is an Epstein–Barr virus (EBV)-relevant human cancer with a unique geographical and ethnic distribution.¹–³ NPC is the most prevalent head and neck malignant disease in the southern of China, with an annual incidence rate of 25–50 cases per 100000 people in this area.⁴–⁶ Owing to the distinct sensitivity to radiotherapy,⁷–⁹ the control of NPC in early stage (I–II) with radical radiotherapy alone or in combination with concurrent chemotherapy is generally satisfactory.⁹–¹⁰ Unfortunately, 60–70% NPC patients are diagnosed at advanced stage (III–IV) with loco-regional lymph node metastases.¹¹ The treatment of the advanced NPC with radiotherapy and chemotherapy is still effective.¹² However, the occurrences of loco-regional and distant failures during a 5-year period after the standard chemoradiotherapy, which are predicted in approximately 20% of the patients at advanced stage, not only suggest a poor prognosis but also remain a primary cause of death in NPC patients.¹³,¹⁰

Pathogenesis of NPC is highly associated with EBV infection. Aberrant expressions of EBV-encoded latent membrane proteins (LMPs), including LMP1 and LMP2, facilitate the progress of NPC.¹¹,¹² As a functional oncoprotein, EBV-LMP2 empowers NPC cells with various malignant properties, such as the deregulations of cell proliferation and differentiation, the ability to proceed to epithelial-to-mesenchymal transition (EMT) and the resistance to interferon (IFN)-induced antiviral effects.¹¹ On the other hand, the presence of these EBV-encoded proteins offers a potential target for viral antigen-specific cancer immunotherapy in NPC. Indeed, our previous study demonstrated that the dendritic cells (DCs) transfected with recombinant serotype 5 adenoviruses (rAd5) carrying EBV-LMP2 significantly increased the antigen-specific T cell proliferation and therefore enhanced the immune cytotoxicity against NPC cells.¹³ Furthermore, we found that the immunization with rAd5-EBV-LMP2 vaccine stimulated EBV-antigen specific cellular immune response in vivo.¹⁴ Thus, inoculation of rAd5-EBV-LMP2 vaccine may serve as an adoptive approach to stimulate EBV-antigen specific immune cytotoxicity, thereby enabling an effective immunotherapy for NPC. However, the safety and tolerance of vaccination of rAd5-EBV-LMP2 still remained unclear.

Herein we carried out a non-controlled phase I clinical trial to evaluate the safety of rAd5-EBV-LMP2 vaccine in patients with regional advanced NPC. The effects of rAd5-EBV-LMP2 vaccine on peripheral CD³⁺CD⁸⁺ and CD³⁺CD⁸⁻ cells was also determined to estimate the potential immunological effects by the vaccine.

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Results

Baseline Characteristics The demographic and clinical features of the 24 Chinese patients with NPC are shown in Table 1. The mean age was 48±7.2 years. Nineteen patients were male and 5 were female. All the patients had advanced local disease (T3–T4, N0–N1, M0) and/or lymph node metastasis (T1–T2, N2–N3, M0). All of the enrolled patients had received prior radiotherapy. In addition, 79.1% (19/24) of the patients also had treated with concurrent chemotherapy. All the patients had completed the previous radiotherapy/chemotherapy for more than 12 weeks prior to the vaccine inoculation. All the patients were clinically confirmed to be in remission on entry into the trial.

Safety and Tolerance Each of 24 participants completed four vaccinations at the predesigned dose. The trial proceeded through the three levels of increasing vaccine dose ($2 \times 10^9$–$2 \times 10^{11}$ vp/vaccination) with no evidence of dose-limiting adverse reaction. The vaccination-related adverse events are summarized in Table 2. All the vaccine inoculation-related toxicities presented as grade I/II adverse reactions. The most frequent systemic adverse reactions were fatigue (33.0%, 8/24), myalgia (29.2%, 7/24) and cough (29.2%, 7/24), other

<table>
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<th>Patient No.</th>
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<th>Stage/Regimen of chemotherapy</th>
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<td>F</td>
<td>IV (T4N1M0) 72 Gy (NP)</td>
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a) Patient identifiers are indicated by the prefix $xx$–$yy$, where $xx$ denotes the dose level from 01 to 03 and $yy$ denotes the patient number from 01 to 24. b) According to Union for International Cancer Control (UICC) 2010 cancer staging manual. NP, nasopharynx; LN, lymph node; NA, not available.
systemic adverse reactions included leukopenia (12.5%, 3/24), cephalalgia (20.8%, 5/24), drowsiness (20.8%, 5/24), influenza (12.5%, 3/24) and nausea (4.2%, 1/24). The regional adverse reactions were tenderness (54.2%, 13/24), rash (8.3%, 2/24) and pruritis (4.2%, 1/24). In general, the inoculation-related toxicities at all the dose levels were mild and tolerable.

**Effects of rAd5-EBV-LMP2 Vaccine on Peripheral CD$^+$CD$^+$ and CD$^+$CD$^+$ Cells**

The rAd5-EBV-LMP2 vaccine at the low dose ($2 \times 10^9$ vp) or middle dose ($2 \times 10^{10}$ vp) could not continuously change the proportion of peripheral CD$^+$CD$^+$ cells in comparison to the baseline, which was determined before the first vaccine inoculation (D$_0$). However, the rAd5-EBV-LMP2 vaccine at the high dose ($2 \times 10^{11}$ vp) showed continuous elevating-effect on circulating CD$^+$CD$^+$ cells for at least 44 d (D$_{14}$ to D$_{58}$) (Fig. 1A). As compared with baseline value (D$_0$), the average levels of peripheral CD$^+$CD$^+$ cells after vaccine inoculation (D$_7$, D$_{14}$, D$_{28}$, D$_{35}$, D$_{58}$) was significantly increased in 66.6% (6/9) of the patients in high-dose group, but only in 16.7% (1/6) and 22.2% (2/9) of the patients in low-dose and middle-dose group, respectively. These patients were considered to be the cases who had CD$^+$CD$^+$ response. The rAd5-EBV-LMP2 vaccine at any dose did not significantly affect the level of peripheral CD$^+$CD$^+$ cells at each dose (Fig. 1B). Only 11.1% (1/9) of the patients in high-dose group had CD$^+$CD$^+$ response, while none of the patients in low-dose (0/6) or middle-dose (0/9) group had CD$^+$CD$^+$ response.

**Follow-Up after Vaccine Inoculation**

Of the 24 enrolled patients, four patients experienced disease-progression events within the two-year follow-up after rAd5-EBV-LMP2 vaccine inoculation (Fig. 2A). The most common progression event was distant failure (12.5%, 3/24) (Fig. 2B), and one patient suffered loco-regional failure (4.2%, 1/24). At the end of follow-up, no patient died for NPC and all the patients remained alive (100%, 24/24).

![Fig. 1](image1.png)

**Fig. 1.** Effects of rAd5-EBV-LMP2 Vaccine on Circulating CD$^+$CD$^+$ and CD$^+$CD$^+$ Cells

Changes of proportion of CD$^+$CD$^+$ (A) and CD$^+$CD$^+$ cells (B) in peripheral blood are shown. Data are presented as the mean±S.D. *p<0.05 vs. the baseline (D$_0$) in each group.

![Fig. 2](image2.png)

**Fig. 2.** Kaplan–Meier Survival Curves of Progression-Free Survival (A) and Distant Failure-Free Survival (B)

The status of progression-free survival and distant failure-free survival in the NPC patients inoculated with rAd5-EBV-LMP2 vaccine are shown.
Discussion

The present study aimed to estimate the safety and tolerance of rAd5-EBV-LMP2 vaccine in patients with advanced NPC. In general, healthy volunteers should be included for safety assessment in a phase I clinical trial. However, LMP2 is an EBV-encoded functional oncoprotein, although the vaccine was replication-defective, the long-term outcome of rAd5-EBV-LMP2 vaccination in healthy volunteers is unpredictable, and therefore only the patients with regional advanced NPC were enrolled in view of ethical considerations. The trial was designed as a single-ascending-dose study with the limitation of the dose-increasing procedure. Through the three levels of vaccine dose (2×10^6–2×10^11vp/vaccination), no severe adverse event (SAE) was observed. Additionally, all the vaccination-associated toxicities presented as mild adverse reactions (grade I/II). These results thus indicated that the rAd5-EBV-LMP2 vaccination was safe and tolerable as the related toxicities observed in this study were generally mild.

In the present study, high frequency tenderness was found in the patients inoculated with high-dose vaccine, which suggest that this regional adverse reaction was induced in a dose-dependent manner. It might be attributable to the fact that severity of the regional inflammatory response depended on the amount of the injected exogenous adenoviruses. Thus this regional adverse reaction should be carefully monitored in the subsequent trials. On the other hand, the incidence of systemic adverse reactions was found to decrease with the increasing dosage of vaccine (Table 2). Since our study was a phase I clinical trial and the patients were sequentially enrolled, the characteristics of patients among three dose groups were unbalanced. The unbalanced demographic distribution thus might interfere into the tolerability of the vaccine, thereby resulting in the inexplicable result described above.

Concurrent radiotherapy and chemotherapy have been established as the standard treatment for the loco-regionally advanced NPC.15) However, the adjuvant chemotherapy after the standard treatment could not provide additional benefit for the patients with advanced NPC in a phase III multi-center randomized controlled trial.10) In view of the frequently overexpressed epidermal growth factor receptor (EGFR) in NPC, utilization of the monoclonal antibody against EGFR may be another candidate strategy for the treatment of advanced NPC.15) In addition, the effectiveness and outcome of other optional targeted therapies, including anti-angiogenic agents, AKT inhibitors and epigenetic approaches, still remain controversial.15)

NPC is a unique human malignancy for not only its extremely unbalanced worldwide distribution but also the EBV-relevant pathogenesis.1–3) In theory, the non-host EBV-proteins offer a natural target for self-driven immune elimination, such as the cytotoxic T lymphocytes (CTLs)-mediated immune response. However, it was found that CTLs-mediated immune reaction was suppressed in EBV-relevant malignances as compared to the healthy EBV-carriers.16,17) Sing et al. showed that the malignant Reed–Sternberg (R–S) cells expressing EBV-LMP2 were susceptible to be lysed by CD^8^+ CTLs, and LMP2-specific CTL clones could be generated from individual Hodgkin’s disease (HD) patients by cloning from the polyclonal EBV-reactive T-cell or direct stimulation of peripheral blood mononuclear cells (PBMC) with the cells expressing LMP2.18) These results strongly suggested that the CTLs-mediated immune response to viral antigens were an achievable anti-tumor treatment for EBV-relevant cancers. Since then intensive efforts have been focused on the development of LMP2-specific CTLs-mediated immunotherapy for EBV-positive human tumors. An adenoviral vector-based vaccine, AdE1-LMPpoly, which encodes a poly-epitope of defined CD^8^+ T cell epitopes from LMP1 and LMP2 fused to Gly-Ala repeat-deleted EBV nuclear antigen-1 (EBNA1), was shown to be an effective immunotherapy for NPC patients with loco-regional or distant metastases, and might bring clinical benefit in a small-sample formal clinical assessment.19) Another phase I trial also reported the safety and immunological effect of a recombinant vaccinia virus termed MVA-EL encoding an EBNA1/LMP2 fusion protein.20) We have developed and established a vector of recombinant serotype 5 adenoviruses carrying EBV-LMP2 (rAd5-EBV-LMP2) to deliver the EBV-antigen.13,14) The rAd5-EBV-LMP2 exhibited promoting CTLs-mediated immune response and anti-tumor effect in vitro and in a mice model.13,14) Therefore, the present first-in-human study was designed to examine the safety and tolerance of rAd5-EBV-LMP2 vaccine in NPC patients.

Li et al. previously showed that the increase of CD^3^+CD^4^+ cells in tumor infiltrating lymphocytes (TILs) was attributable to the EBV-LMPs expressed in tumor tissues.21) Consistently, in this study we found that the intramuscular inoculation of rAd5-EBV-LMP2 vaccine at the high dose (2×10^11vp) obviously increased the circulating CD^3^+CD^4^+ cells (Fig. 1A). CD^4^+ T cells function as mature T-helper cells and play an important role in modulating immune responses to pathogens and tumor cells.22) The increase of circulating CD^4^+ T cells presumably resulted from the immune stimulation activated by major histocompatibility complex (MHC) class II-restricted tumor antigen (EBV-LMP2).22) suggesting the rAd5-EBV-LMP2 vaccine effectively generated antitumor immunity by affecting CD^4^+ T cells. Although our earlier work has demonstrated that the LMP2-specific CTLs consisted of CD^4^+ T cells,13) the phenotypic analysis of human samples should be performed in following study to verify the elevated CD^4^+ cell in NPC patients can represent he EBV-LMP2-specific T cells, and the potential clinical benefit of the elevated circulating CD^4^+ T cells in NPC patients still required to be further clarified as well. On the other hand, various earlier studies suggested that CD^8^+ CTLs-mediated immunity is another dominated EBV-specific immune response to exert anti-tumor effects in NPC.20,23,24) In the present study, rAd5-EBV-LMP2 vaccine at any dose level failed to significantly affect peripheral CD^8^+ T cells. It might be attributed to the lower antigenicity of rAd5-EBV-LMP2 vaccine, which only carried a single EBV-antigen (LMP2), but not the multiple EBV-antigens fusion as described previously.20,23)

During our follow-up after the rAd5-EBV-LMP2 vaccine inoculation, of the 24 NPC patients enrolled in our study, disease-progression events were reported in four patients (16.6%, 4/24) and accordingly the failure-free rate was 83.3% (20/24) at the end of the follow-up for two years. The result seemed to be consistent with the findings in a large-sample phase III trial (84%) in which the NPC patient had similar treatment history.29) In addition, according to the data revealed from another undergoing trial performed in our institution, the failure-free rate was approximately 80% in the patients who had received standard concurrent radiotherapy and chemother-
apy (unpublished data). However, the actual clinical benefit of rAd5-EBV-LMP2 vaccine should be further determined in randomized placebo-control phase II trial and the retrospective analysis of the data from long-term (five-year) observation in the near future.

To summarize, our results indicated that the rAd5-EBV-LMP2 vaccine was safe and well-tolerated. We propose that the high dose \((2 \times 10^{11} \text{vp})\) is an appropriate dose to be adopted in following phase II trials for not only the continuous immunogenic effect but also the safety that confirmed in the present study.

**Experimental**

**Study Design** The present study was a single-center, non-randomized, open-label, single-arm and single-ascending-dose phase I trial. A total of 24 patients were enrolled into the trial from September 2012 to March 2013 at The People’s Hospital of Guangxi Autonomous Region. All the patients recruited in this study provided written informed consent. The trial was approved by the ethics committee of The People’s Hospital of Guangxi Autonomous Region (Approved No. of ethic committee: GCP-2012-18). The trial was registered in Chinese Clinical Trial Registry and the registration number is ChiCTR-ONC-12002502.

The inclusion criteria for the enrolled patients were as follow: 1) Histopathologically confirmed diagnosis of NPC, and any of the following conditions found: advanced local disease (T3–T4, N0–N1, M0) or lymph node metastasis (T1–T2, N2–N3, M0); 2) Post-chemotherapy and radiotherapy ≥3 months, and normal functions of liver, kidney and marrow; 3) Positive immunoglobulin A (IgA) antibody to EBV-viral capsid antigen (VCA); 4) Aged ≥18; 5) Survival duration N3, M0); 2) Post-chemotherapy and radiotherapy any of the following conditions found: advanced local disease; 3) Histopathologically confirmed diagnosis of NPC, and any of the following conditions found: advanced local disease (T3–T4, N0–N1, M0) or lymph node metastasis (T1–T2, N2–N3, M0); 2) Post-chemotherapy and radiotherapy ≥3 months, and normal functions of liver, kidney and marrow; 3) Positive immunoglobulin A (IgA) antibody to EBV-viral capsid antigen (VCA); 4) Aged ≥18; 5) Survival duration ≥3 months; 6) Physical status score Eastern Cooperative Oncology Group (ECOG: 0–1; 7) Informed consents were signed from all patients prior to the study. The exclusion criteria were: 1) Accompaniment by systemic infection, autoimmune disease or any immunodeficiency disease; 2) Pregnant or lactating women; 3) Treatment with systemic corticosteroid; 4) Allergy to drug; 5) human immunodeficiency virus (HIV) positive or active hepatitis B virus infection; 6) Other factors unsuitable for the trials.

**Inoculation of rAd5-EBV-LMP2 Vaccine** The enrolled patients were sequentially assigned into three dose-increasing groups (low dose, \(2 \times 10^{10} \text{vp}\), middle dose, \(2 \times 10^{10} \text{vp}\), n=9; high dose, \(2 \times 10^{10} \text{vp}\), n=9). The replication-defective rAd5-EBV-LMP2 vaccine was intramuscularly injected in upper arm of the patients for four times with a seven-day interval (D0, D6, D14, D28). When grade III/IV systemic adverse events (except for pyrexia) or grade IV regional adverse events were observed in at least 1/3 of the patients in the lower dose group, the trial was not allowed to proceed to the next higher dose assignment.

**Detection of Peripheral CD4^+CD8^+ and CD8^-CD4^- Cells** The levels of peripheral CD4^+CD8^- and CD4^-CD8^+ cells were examined to estimate the immunological effects of rAd5-EBV-LMP2 vaccine in the NPC patients. The peripheral blood samples were collected prior to each vaccine inoculation (D0, D6, D14, D28), as well as 7 and 30d after the last inoculation (D35, D58). The percentages of CD4^+CD8^- and CD4^-CD8^+ cells accounted for the proportions of peripheral blood mononuclear cells were determined, respectively, by a flow cytometry (FCM) in the Clinical Laboratory Center of our institution according to standard protocol. The average levels of peripheral CD3^+CD4^-/CD3^+CD8^- and CD4^-CD8^+ cells after vaccine inoculation were evaluated by calculating the mean of all the value determined at each time-point post the inoculation (D0, D6, D14, D28). The patient who with a significantly increased average level of peripheral CD3^+CD4^-/CD3^+CD8^+ cells compared with the value determined before the inoculation (D0) was defined as a case who had CD3^+CD4^-/CD3^+CD8^- responses, respectively.

**Follow-Up** The enrolled patients were required to return to our institution for a routine assessment every three months after the vaccine inoculation. High-definition electronic endoscopy, whole-body bone Emission Computed Tomography (ECT), nasopharynx and skull base magnetic resonance imaging (MRI) were performed to review the relapse-free or progression-free status in the NPC patients. In addition, the contact was also made by the follow-up division in our institution every month through the registered telephone. Anything about the change of disease progression was documented in detail.

**Statistical Analysis** The graphical values of proportion of peripheral T cells were shown as the mean±standard deviation (S.D.). Paired Student’s t-test was used for statistical analyses. Statistical significance was assumed at \(p<0.05\) and indicated with asterisk.

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**Conflict of Interest** The authors declare no conflict of interest.

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