A phase II study evaluating the safety and efficacy of an adenovirus-ΔLMP1-LMP2 transduced dendritic cell vaccine in patients with advanced metastatic nasopharyngeal carcinoma

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Received 1 December 2010; revised 20 April 2011; accepted 30 May 2011

Background: Individuals with metastatic Epstein–Barr virus (EBV)-positive nasopharyngeal carcinoma (NPC) continue to have poor outcomes. To evaluate the ability of a dendritic cell (DC) vaccine to target subdominant EBV antigens LMP1 and LMP2 expressed by NPC cells, we vaccinated patients using autologous DCs transduced with an adenovirus encoding a truncated LMP1 (ΔLMP1) and full-length LMP2 (Ad-ΔLMP1-LMP2).

Materials and methods: Sixteen subjects with metastatic NPC received Ad-ΔLMP1-LMP2 DC vaccines i.d. biweekly for up to five doses. Toxicity, immune responses and clinical responses were determined.

Results: Most patients had extensive disease, with a median of three visceral sites of involvement (range 1–7). No significant toxicity was observed. Ad-ΔLMP1-LMP2 DCs induced delayed type hypersensitivity responses in 9 out of 12 patients, but although these DCs activated LMP1/2-specific T cells in vitro, no such increase in the frequency of peripheral LMP1/2-specific T cells was detected. Three patients had clinical responses including one with partial response (for 7½ months) and two with stable disease (for 6½ and 7½ months).

Conclusions: Ad-ΔLMP1-LMP2 transduced DCs can be successfully generated and safely administered to patients with advanced NPC. Since efficacy was limited, future studies should focus on DC vaccines with greater potency administered to subjects with less tumor burden.

Key words: dendritic cell vaccine, Epstein–Barr virus, nasopharyngeal carcinoma, recombinant adenovirus

introduction

Nasopharyngeal carcinoma (NPC) is an epithelial tumor that is common in South China and Southeast Asia, where incidence reaches ~25 per 100 000 [1, 2]. The outcome for patients with advanced-stage disease remains poor and those who survive long term often suffer from severe treatment-related morbidity [3]. Thus, there is a need to develop therapies, which reduce mortality from the disease and suffering from the treatment.

Since all distinct histological subtypes of NPC are universally associated with Epstein–Barr virus (EBV), one promising approach is to target the EBV antigens EBNA1, LMP1 and LMP2 expressed in NPC [4, 5]. While the transfer of autologous EBV-specific cytotoxic T lymphocytes (CTLs) has shown promising results in phase I/II clinical studies [6–9], broader application is limited by the lengthy production process (12–18 weeks) and by the dominance of T cells within the resulting CTL lines, which are largely specific for immunodominant EBV antigens that are absent from NPC [10]. As an alternative, immune responses could be produced in vivo using vaccines directed to NPC-associated EBV antigens [11, 12]. So far, the experience with EBV-targeted vaccines is limited. In one study, 16 patients with advanced NPC were vaccinated with dendritic cells (DCs) loaded with LMP2 peptides [13]. Peptide-specific T-cell responses were elicited or boosted in nine patients and a partial tumor reduction was observed in two patients. An independent vaccine study administers a recombinant vaccinia virus expressing an EBNA1-LMP2 fusion protein to patients with the disease [14, 15].

We have constructed a recombinant Ad5Δ535 virus encoding an inactive form of LMP1 (ΔLMP1) and full-length LMP2...
(Ad-ΔLMP1-LMP2). To evaluate the clinical and immunologic effects of a DC vaccine transduced by this Ad-ΔLMP1-LMP2, we conducted a phase II clinical study of these cells and now report our results.

materials and methods

study design and patients
This prospective, open-labeled single-center phase II study was approved by the Institutional Review Board of the National Cancer Centre, Singapore. The primary objective was to determine the clinical benefit rate—complete response (CR), partial response (PR) and stable disease (SD)—of longer than 14 weeks, following five injections of autologous Ad-ΔLMP1-LMP2 DC vaccine. Response was evaluated by computer tomography using RECIST. Secondary objectives were tolerability, progression-free survival (PFS) and overall survival (OS). Patients with histologically proven, EBV-positive metastatic NPC (World Health Organization type II/III) were eligible for the study if they had progressed on at least one line of polychemotherapy and had received no therapy or experimental agents for at least 4 weeks before vaccine administration. Patients were excluded if they had active or severe cardiac, pulmonary or cerebrovascular disease. In addition, patients with a creatinine clearance of <40 ml/min, serum bilirubin of >1.5x the upper limit of normal, or ALT or AST >5x the upper limit of normal were excluded, as were patients with active uncontrolled infection, active central nervous system disease or positive serology for human immunodeficiency virus or Hepatitis B or C.

Ad-ΔLMP1-LMP2 virus
The Ad-ΔLMP1-LMP2 virus is a replication-incompetent adenovirus, which has serotype 5 capsid containing the short-shafted fiber protein of serotype 35 (Ad5S35; supplemental Figure S1, available at Annals of Oncology online). It was generated using the pShuttleX system (BD Biosciences, Palo Alto, CA) as previously described [16–18]. Clinical grade vector was produced at the Center for Cell and Gene Therapy, Baylor College of Medicine (Houston, TX).

vaccine preparation
Autologous DC vaccines were produced under standard Good Manufacturing Practice conditions. Peripheral blood mononuclear cells (PBMC) required for DC vaccine production were obtained from the 16 subjects by one (13/16) or two (3/16) venesection (200–350 ml), followed by Ficoll (GE Healthcare Biosciences, Pittsburgh, PA) density gradient centrifugation. Monocytes were isolated from PBMCs by the adherence method [19, 20] and cultured for 5 days in CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with 100 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF; CellGenix) and 50 ng/ml of interleukin (IL)-4 (CellGenix) to generate immature DCs. On day 5, immature DCs were transduced with the Ad-ΔLMP1-LMP2 vector at a multiplicity of infection of 1 : 1000 and matured with a cytokine cocktail including GM-CSF, IL-4, IL-1β, IL-6, tumor necrosis factor-α and prostaglandin E2 (Sigma, St Louis, MI). On day 7, transduced DCs were harvested. An aliquot of 3–5 x 10^6 cells was used for immediate vaccination, while the remaining vaccines doses cryopreserved in separate 3–5 x 10^6 cell aliquots. Quality controls for the vaccines included sterility tests, phenotyping and viability testing.

fluorescence-activated cell sorter analysis
We immunophenotyped the DCs by multi-color flow cytometric analysis (FACScalibur Becton Dickinson, San Jose, CA) using staining methods previously described [16]. Intracellular staining using anti-LMP1 (CS1-4; Fitzgerald Industries, Acton, MA) and anti-LMP2 (14B7; gift of Dr Grasser, University of Homburg/Saar, Germany) was done as previously described [16, 21].

treatment plan and response assessment
Each patient received up to five i.d. injections of the vaccine in the forearms and deltoids, at biweekly intervals at a dose of 3–5 x 10^6 cells each. Patients on study were reviewed before each vaccination and monthly thereafter. Full blood count, electrolytes and liver enzymes were measured at baseline and before each vaccination. Radiological imaging was carried out at baseline, before the fourth vaccination (week 6) and at weeks 14 and 18. All responses were assessed using RECIST criteria [22]. Patients were followed for clinical benefit, response, PFS and OS and toxic effects were graded according to National Cancer Institute—Common Toxicity Criteria (version 3.0). PBMCs for correlative studies were obtained at baseline, immediately before the second and fourth vaccination and at months 1 and 2 after the final vaccination.

elispot assay
Patients’ PBMCs were isolated using standard Ficoll techniques and cryopreserved. Interferon (IFN)-γ Elispot assays were carried out using a single plate to process samples from multiple time points of a single patient as previously described [9]. Pepmixes of EBV antigens (LMP1, LMP2, EBNA1, EBNA3A), cytomegalovirus (CMV) antigens (pp65, IE1) and adenovirus antigens (penton, hexon) were used in the Elispot assays. These contained 15 amino-acid peptides covering the entire length of the corresponding protein with an 11 amino-acid overlap (JPT Peptide Technologies, Germany). Developed Elispot plates were analyzed (ZellNet Consulting). Spot-forming cells (SFCs) per PBMCs were calculated and expressed as SFC per 10^7 PBMCs.

in vitro dendritic cell coculture assays
Autologous LMP1/2-expressing DCs were co-incubated with patient’s prevaccination PBMCs in 24-well plates at a ratio of 1 : 10 in culture media (90% RPMI-1640, 10% FBS, 2 mmol GlutaMAX-I). After 8–10 days, cells were analyzed for LMP1- and LMP2-specific responses using Elispot assays.

delayed type hypersensitivity response
Patients received i.d. injections of transduced and nontransduced autologous DCs (0.5 x 10^6 cells each) at separate sites on the forearm at the third and fifth vaccination. Delayed type hypersensitivity (DTH) was assessed by determining the diameter of skin induration at 48 h post injection. A positive reaction was defined as ≥25 mm induration at the site of transduced DC injection and ≥23 mm larger than the control DC site.

plasma EBV-DNA analysis
For EBV-DNA measurements, DNA was extracted from 800 µl of plasma and amplified by RT-PCR (Applied Biosystems, Carlsburg, CA). Cycling conditions and analysis have been previously described [23].

statistical analysis
Exploratory analyses comprising of graphical and quantitative techniques were used to analyze the data of the clinical trial. Overall best response rate was assessed according to clinical benefit as defined by CR, PR or SD for a minimum of 14 weeks, along with the corresponding 95% confidence interval (CI) using the Wilson method. Kaplan–Meier survival curves were used to analyze the OS and the PFS of the enrolled patients. The duration of OS was defined from the date of registration to death from any cause, or last follow-up. PFS was measured from the date of registration to the date of documented disease progression, death from any cause, or last follow-up.
Differences in OS between low and high EBV groups were compared using the log-rank test. Analyses were done in SAS 9.2 and $P \leq 0.05$ was considered significant.

**results**

**patient characteristics**

Between August 2007 and September 2008, we screened 25 patients with metastatic NPC and consented and enrolled 16 into this study. All patients were Asian with the majority Chinese (87.5%) and male (81%), with a median age of 49.7 years (range 36–58 years). Most patients had extensive disease, with a median of three visceral sites of involvement (range 1–7), and all had documented progression of disease before entry into the study. Patients were also heavily pretreated, with median 3.5 lines of prior chemotherapy (range 1–8) and median 4.5 number of agents used (range 1–8). All patients except one had prior radiotherapy to the posterior nasal space and neck, and four had additional radiotherapy to symptomatic bony sites. Median Eastern Cooperative Oncology Group (ECOG) performance status was 1, with 4 patients with ECOG 0, 10 with ECOG 1 and 2 with ECOG 2. All patients had

**Figure 1.** Characterization of dendritic cell (DC) vaccine. A) DC vaccine phenotype showed robust MHC class II, CD83 and CD86 expression for the majority of patients. B) LMP1 and C) LMP2 expression after Ad-LMP1-LMP2 transduction as judged by fluorescence-activated cell sorter analysis. DCs from patients D) 008 and E) 012 were cocultured with autologous PBMCs. Interferon-γ ELispot analysis carried out on day 10 revealed reactivation of LMP2- and adenovirus (penton/hexon)-specific T cell in both patients and LMP1-specific T cells in one out of two patients. MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells.
documented progressive disease when they entered on the study.

**DC vaccine characterization**

Both fresh (first dose) and frozen DC vaccines (subsequent doses) were analyzed using flow cytometry. Analysis of 15 fresh DC vaccines demonstrated expression of major histocompatibility complex (MHC) class II molecules (median 97.2%; range 76.7%–100%), the costimulatory molecules CD86 (median 88.5%; range 47.9%–99.8%) and CD83 (median 56.7%; range 20.6%–90%), and CCR7 (median 22.2%; range 5.7%–56.6%; Figure 1A). Thawed DC vaccines had a similar phenotype (data not shown). Intracellular staining of fresh and thawed DC for LMP1 and LMP2 showed high levels of expression of both transgenes (Figure 1B and C).

To determine whether the LMP1/2-expressing DCs activated LMP1- and LMP2-specific T cells in vitro, patients’ DCs were cocultured with autologous PBMCs. IFN-γ Elispot analysis on day 10 showed an increase of LMP2-specific T cells in both patients and for LMP1 in comparison to baseline in one patient. In addition, an increase of adenovirus-specific T cells was observed in both patients. These results indicate that the DC vaccines were able to activate LMP1- and LMP2-specific T cells even from heavily pretreated NPC patients (Figure 1D and E).

**safety of DC vaccine administration**

A total of 64 vaccines injections were administered to 16 subjects, 9 of whom completed the full five vaccinations. Patients who failed to complete vaccination had rapid disease progression. The median dose per vaccine was $4.82 \times 10^6$ DCs (range 3.69–5.06 $\times 10^6$ cells) and the median dose per patient was $22.7 \times 10^6$ cells (range 3.69–25 $\times 10^6$ cells). All patients tolerated treatment without significant side-effects. Mild nonhematological toxic effects such as fever, fatigue and skin rash occurred in nine patients (grade 1; supplemental Table S1, available at Annals of Oncology online). Grade 1/2 and grade 3 anemia was seen in 14 patients (87.5%) and 1 patient (6.3%), respectively. These were considered related to the underlying disease.

**immune responses after DC vaccine**

We used DTH responses as a surrogate marker to assess vaccine-induced immune responses. In addition, we determined the precursor frequency of LMP1- and LMP2-specific T cells in patients’ peripheral blood. DTH tests were carried out in 12/16 patients (Figure 2). Positive reactions to transduced DCs were seen in 9 (75%) out of 12 patients at the time of the third vaccination with a median induration of 8 mm (range 5–13 mm). Positive DTH reactions were also observed in 4/8 patients after the fifth vaccination, but their magnitude was smaller (median 6 mm; range 6–7 mm). Of the eight patients, three evaluated twice had positive reactions at both time points.

We used IFN-γ Elispot analysis to determine the precursor frequency of antigen-specific T cells in patients’ PBMCs. Before vaccine, 3/11 tested patients had low responses (<50 SFC/10^5 cells) to the positive control (Staphylococcus aureus toxin), indicating global immunosuppression.

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**Figure 2.** Dendritic cell vaccines induce delayed DTH responses. DTH tests were carried out on at baseline, third vaccination (12 patients), and fifth vaccination (eight patients). A positive test was defined as induration >5 mm, (and >3 mm greater than control) at 72 h and was observed in nine patients at the third vaccination (A) and in four patients at the fifth vaccination (B). DTH, delayed type hypersensitivity; PR, partial response; SD, stable disease.
(Figure 3A). Of the eight patients, five with normal *S. aureus* toxin responses had responses to the immunodominant CMV antigens, indicating the presence of functional viral-specific T cells. No patient had responses to EBV-LMP1 or LMP2, while two patients had responses to EBNA1 (Figure 3B). After vaccination, we did not observe an increase in the frequency of LMP1/2-specific T cells in any of the eight patients analyzed, and there was no increase in the frequency of adenovirus-specific T cells.

**clinical responses and survival**

The group’s median PFS was 1.92 months (95% CI 1.6–2.1 months) and the median OS time was 6.0 months (95% CI 2.73–11.7 months; Figure 4). The 6-month and 1-year survival rates were 50% and 19%, respectively. One patient (6%) achieved a PR for 7½ months, and two (12.5%) others had SD for 6½ and 7½ months (Table 1). The patient who achieved PR was a 46-year-old male who had extensive disease in the cervical lymph nodes, posterior nasal space and chest wall and had received prior chemotherapy with paclitaxel, gemcitabine and carboplatin. Approximately 2 months after the fifth vaccination, he achieved a PR with cavitary necrosis of the tumor in his posterior cervical neck node (Figure 5).

We measured EBV-DNA load in peripheral blood since this is a promising biomarker for NPC disease burden [24]. Five of 12 with low EBV-DNA load (<500 copies per ml) had a statistically longer OS than patients with a high EBV-DNA load.
In this study, we evaluated the safety and efficacy of an Ad-DLMP1-LMP2 transduced DC vaccine in patients with extensively pretreated metastatic NPC. We show that even from these extensively pretreated patients, it is feasible to routinely generate autologous DC vaccines, which express high levels of MHC class II molecules, costimulatory molecules, LMP1 and LMP2 and are capable of activating LMP1-, LMP2- and adenovirus-specific T-cell responses in vitro. While we observed DTH responses in 9 out of 12 patients post vaccination in vivo, no LMP1-, LMP2- or adenovirus-specific T-cell responses were detected in the peripheral blood. DC vaccination was associated with tumor response or SD in 3/16 patients as judged by imaging.

For the expression of LMP1 and LMP2 in DCs, we constructed a recombinant Ad containing LMP1, starting at amino-acid 44 to render it functionally inactive [16] and full-length LMP2 (Ad-DLMP1-LMP2). Full-length proteins were chosen to allow the activation of CD4- and CD8-positive LMP1/2-specific T cells regardless of the patients’ human leukocyte antigen (HLA) type. Characteristics and in vitro functionality of the transduced DCs are discussed in detail in the online available supplemental text (available at Annals of Oncology online).

Although we detected no rise in the frequency of LMP1-, LMP2- or adenovirus-specific T cells responses post vaccination, the detection limit of Elispot assays is 1/50 000 and only 2% of the entire lymphocyte pool resides in the peripheral blood [25, 26]. But even if a low-level immune response is induced by the modified DC, it is clearly well below the levels desired for clinical effectiveness. To elucidate the reason for the limited efficacy of the DC vaccine, it would have been instructive to generate LMP1- and LMP2-specific T cells from the enrolled NPC patients by stimulating their PBMCs with LMP1 and LMP2 peptides. Due to limited patients’ material, we were unable to perform these studies. Nonetheless, groups have shown that LMP1- and/or LMP2-specific T cells are present in the majority of NPC patients albeit at low frequency [27]. In addition, we detected in 4/32 EBV-specific CTL lines generated from NPC patients with metastatic disease at our center patients LMP1-specific T cells were as LMP2-specific T cells were present in 22/32 CTL lines [28].

Limited efficacy of a DC vaccine in NPC patients was also observed in one published prior clinical study. Sixteen patients with recurrent or metastatic NPC received four weekly injections of DCs pulsed with HLA-A1101, A2402 or B4011 restricted LMP2 peptides. LMP2-specific T-cell responses were transiently elicited or boosted in nine patients post vaccination at a very low frequency (6–12 LMP2-specific T cells/10⁵ PBMCs) [13].

Although systemic, tumor-mediated, immunosuppression might have contributed to the limited efficacy of these DC vaccines, the NPC patients on our study did not have an increased frequency of inhibitory regulatory T cells in their peripheral blood (Supplemental Figure S3A, available at Annals of Oncology online) [29]. We also saw no increase in systemic levels of the immunosuppressive cytokines IL-10 or transforming growth factor-β (Supplemental Figures S3B and C, available at Annals of Oncology online). Thus, while our results make systemic immunosuppression less likely, we cannot exclude immunosuppression within the local tumor microenvironment, which is described for NPC [30, 31].

The DC vaccine dose in this study was 3.69–5.06 × 10⁶ cells. If a higher DC vaccine dose would have increased, efficacy needs to be addressed in a future clinical trial. Besides increasing cell dose, the efficacy of DCs might also be enhanced by genetically modifying them [32–34]. Alternatively, the combination of T-cell transfer with vaccination may be beneficial even when vaccination or T-cell transfer alone is ineffectual [35].

Of the 16 patients treated, 3 had clinical responses including 2 patients with SD and 1 with a PR. Unlike melanoma and renal cell carcinoma, spontaneous remissions do not occur in advanced NPC [36, 37]. All patients had documented cancer progression before entry into the study. Thus, the finding of SD or a PR for 6½–7½ months would generally be accepted as clinically ‘promising’, albeit at a lower level of clinical significance than more dramatic tumor responses.

Figure 4. Kaplan–Meier survival graph. A) Progression-free survival and B) overall survival of patients post dendritic cell vaccination.
The response rate in our study is similar to that observed in the prior EBV peptide DC vaccine study in which 2/16 patients had a PR [13]. In meta-analyses of >170 DC vaccine studies for patients with solid tumors, the overall response rate was <5% [38–40]. To date, only one DC cancer vaccine for hormone-refractory prostate cancer, Sipuleucel-T, has been proven to improve survival in a randomized phase III setting; interestingly, no PFS benefit was noted. This finding indicates

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BOR, best overall response; BOS, base of skull; Carbo, carboplatin; CDDP, cisplatin; DC, dendritic cell; F, female; Gem, gemcitabine; LN, lymph node; M, male; OS, overall survival; Pac, paclitaxel; PD, progressive disease; PFS, progression-free survival; PNS, posterior nasal space; PR, partial response; PS, performance status according to Eastern Cooperative Oncology Group; RT, radiotherapy; SC, subcutaneous; SD, stable disease; unk, unknown; 5FU, 5-flourouracil.
that while PFS is still a valid end point for studies like ours, it may underestimate the true benefit of therapeutic vaccines. We have shown that DC vaccines can be successfully generated using Ad-ALMP1-LMP2 even from heavily pretreated NPC patients with extensive disease and induce modest clinical responses. Although the potency of the current vaccine is too low for significant benefit in patients with extensive disease, further modifications to the DC, combination with other cellular immunotherapies and choice of patients with lower disease burden may all serve to improve future effectiveness.

acknowledgements

We thank Malcolm K. Brenner for the helpful discussion and advice.

funding

Singapore Cancer Syndicate (SCS-TS0027); National Institute of Health grant (2P01 CA94237 to SG).

disclosure

The authors declare no conflicts of interest.

references


