Adoptive T-cell Transfer and Chemotherapy in the First-line Treatment of Metastatic and/or Locally Recurrent Nasopharyngeal Carcinoma

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The outcomes for patients with metastatic or locally recurrent Epstein–Barr virus (EBV)-positive nasopharyngeal carcinoma (NPC) remain poor. Adoptive immunotherapy with EBV-specific cytotoxic T lymphocytes (EBV-CTLs) has proven clinical efficacy, but it has never been evaluated in the first-line treatment setting in combination with chemotherapy. To evaluate the safety and efficacy of a chemotherapy in combination with adoptive EBV-CTL transfer, we conducted a phase 2 clinical trial consisting of four cycles of gemcitabine and carboplatin (GC) followed by up to six doses of EBV-CTL. Thirty-eight patients were enrolled, and 35 received GC and EBV-CTL. GC-CTL therapy resulted in a response rate of 71.4% with 3 complete responses and 22 partial responses. With a median follow up of 29.9 months, the 2-year and 3-year overall survival (OS) rate was 62.9 and 37.1%, respectively. Five patients did not require further chemotherapy for more than 34 months since initiation of CTL. Infusion of CTL products containing T cells specific for LMP2 positively correlated with OS (hazard ratio: 0.35; 95% confidence interval: 0.14–0.84; *P* = 0.014). Our study achieved one of the best survival outcomes in patients with advanced NPC, setting the stage for a future randomized study of chemotherapy with and without EBV-CTL.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial cancer etiologically linked to Epstein–Barr virus (EBV). Although rare in Western Europe and North America, NPC is a leading cancer in Southern China and Southeast Asia with an incidence rate of 10–21.4 per 100,000 males.[1,2] Although conventional therapy with concurrent chemoradiation therapy achieves a high cure rate in localized NPC,[3] patients with metastatic disease continue to have a poor prognosis with a median overall survival (OS) of 11–22 months. Although there is no standard of care for patients with metastatic disease,[4] the combination of gemcitabine, carboplatin, and paclitaxel chemotherapy generates one of the highest response and OS rates among palliative regimen.[5,6] Up to date, targeted agents have not been shown to significantly improve clinical outcomes,[7–9] and their role in the treatment of NPC currently remains limited.

Because the majority of NPCs are positive for EBV,[10] targeting EBV antigens expressed in NPC is an attractive approach to improve outcomes for patients with advanced disease. Indeed, adoptive transfer of EBV-specific cytotoxic T lymphocytes (EBV-CTLs) as single-agent therapy has shown clinical benefit in phase 1 and 2 NPC clinical studies.[11–16] However, the majority of patients were treated in the western hemisphere where NPC is sporadic, and studies have included heterogeneous groups of patients who have refractory disease,[16] cancer in remission,[13] or who have received varying lines (between 1–6) of previous salvage chemotherapy.[15] This has made it difficult to accurately assess the clinical benefit of adoptive CTL transfer in NPC patients.

To evaluate whether we could safely combine chemotherapy with adoptive transfer of EBV-CTLs in patients with locally recurrent or metastatic, endemic NPC, we conducted a phase 2 clinical trial in which patients received four cycles of gemcitabine and carboplatin (GC) followed by up to six sequential infusions of EBV-CTLs as first-line therapy (**Figure 1**). This phase 2 study represents the first application of adoptive T-cell therapy in the upfront treatment of any cancer.

RESULTS

Patient characteristics

The clinical and disease-specific characteristics of the 38 patients are summarized in **Table 1**. All patients were Asian, and the majority were male (73.7%) Chinese (94.7%), with a median age of 57 years (range: 27–77 years). Of these, 37 patients (97.4%) had WHO type III NPC. Moreover, 19 patients (50%) had metastatic disease at distant sites, 9 (23.7%) in only locoregional sites, and 10 patients' (26.3%) disease involving both locoregional and distant sites. The median number of involved sites was three.

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Twenty-four patients (63.2%) had an Eastern Cooperative Group (ECOG) performance status of 1, and 14 patients (36.8%) were ECOG 0.

Characteristics of EBV-CTL lines

EBV-CTL lines were successfully generated for 37 of the 38 patients enrolled in the study. The median time taken to produce and release the first dose of CTLs was 13 weeks (range: 8–22 weeks). The generated EBV-CTL lines contained predominately CD8-positive T cells with a mixture of effector memory, late effector memory, and central memory T cells (**Supplementary Figure S1A**). Of 35 cell lines evaluated, T cells specific for immunodominant EBV antigens (BZLF1, BRLF1, BRMF1, or EBNAs 3A, B, C) were present in all the cell lines, LMP2-specific T cells in 26 cell lines, LMP1-specific T cells in 8 cell lines, and EBNA1-specific T cells in 3 cell lines (**Supplementary Figures S1B,S1C**).

Treatment received

Of the 38 patients, 31 patients completed the planned schedule of GC, whereas 3 patients received additional chemotherapy (up to six cycles) due to delays in CTL production. Four patients did not complete induction chemotherapy due to disease progression or death during chemotherapy. Median relative dose intensity of administered gemcitabine and carboplatin were 0.75 (range: 0.33–1.0) and 0.70 (range: 0.33–1.0), respectively.

Thirty-five patients received EBV-CTLs, 24 (68.6%) of whom completed all six cycles. The median number of administered CTL doses was 6, and the median total CTL dose was 9.6×10^8 cells (range: $6.3–10.3 \times 10^8$ cells). Eleven of the 35 patients did not receive the prescribed six CTL doses due to disease progression. For three subjects, CTLs were not initiated due to rapid disease progression (patient 19) or death (patients 18 and 28).

Toxicities

Hematological and nonhematological toxicities are summarized in **Supplementary Table S1**. Immunotherapy with EBV-CTLs was well tolerated. No patients experienced any grade 3, 4, or 5 hematological or nonhematological toxicities during immunotherapy. The most common CTL toxicities were grade 1 and 2 fatigue and grade 1 myalgia. Two patients experienced transient CTL infusion-related fever, and three patients developed grade 1 skin rash.

All grade 3 and above toxicities in this study occurred during chemotherapy. Three patients experienced severe adverse events related to chemotherapy. Patient 28 died of aspiration pneumonia and neutropenic sepsis, and patient 16 was admitted for grade 3 epistaxis secondary to grade 3 thrombocytopenia that was resolved. Patient 18 died after the third cycle of chemotherapy from bacterial meningitis that was contributed by a skull-based tumor invading the brain.

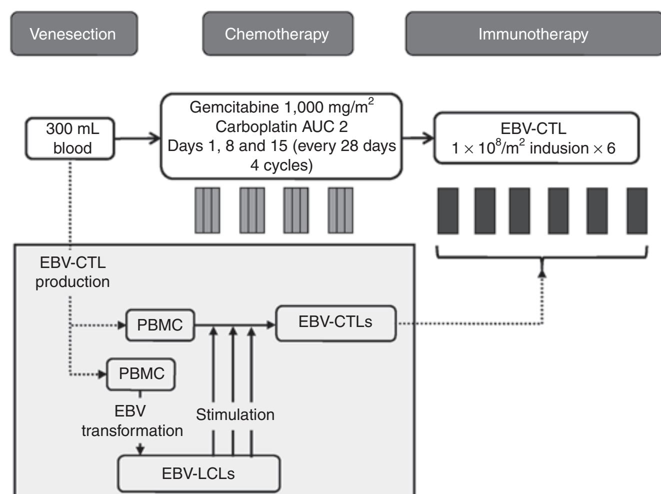

Figure 1 Scheme of clinical trial. For details see text. CTLs, cytotoxic T lymphocytes; EBV, Epstein–Barr virus; LCL, lymphoblastoid cell line; PBMC, peripheral blood mononuclear cell.

Clinical response rates and survival

After a median follow-up of 29.9 months, 12 patients (31.5%) were alive of whom two (5.3%) had no evidence of progressive disease. Out of the 38 patients, who received chemotherapy, 3 patients (7.9%) had a complete response, 21 patients (55.3%) had a partial response, and 12 patients (31.6%) had SD as best response to chemotherapy, leading to a response rate of 63.2% and a clinical benefit rate of 94.7%. One patient (2.6%) progressed on chemotherapy and one (2.6%) was not evaluable (**Table 2**). Response to immunotherapy was determined by comparing pre-CTL (post-chemotherapy) and post-CTL imaging studies with a new tumor baseline established after completion of chemotherapy. Out of the 35 patients who received CTLs after chemotherapy, 2 patients remained in complete response (5.7%), 13 patients had a further partial response (31.7%), and 7 (20%) had SD as best response to CTL therapy, leading to a response rate of 42.9% and a clinical benefit rate of 62.9%.

Of the 35 patients who received chemotherapy and CTL (GC-CTL), the median OS was 29.9 months (95% confidence interval (CI): 20.8–39.3) with 1-, 2-, and 3-year OS rates of 77.1% (95% CI: 59.5–87.9), 62.9% (95% CI: 44.8–76.5), and 37.1% (95% CI: 21.7–52.7), respectively (**Table 3** and **Figure 2a**). The median overall progression-free survival (PFS) was 7.6 months (95% CI: 7.4–8.4), with 25.7% of patients being free of disease progression at the 1-year mark (**Figure 2b**). The median PFS for the CTL immunotherapy phase was 3.7 months (95% CI: 2.4–4.0; range: 2.0–35.3 months).

To determine the impact of CTLs on tumor growth kinetics, we measured the serial changes in tumor size in patients during chemotherapy and immunotherapy (**Supplementary Figure S2A**). Decreased tumor growth rates were observed in several patients, and in at least two patients, tumor shrinkage was noted after initial progression. Five patients (patients 1, 8, 13, 14, and 20) had prolonged disease stabilization of more than 52 weeks after the first CTL infusion. Furthermore, five patients (patients 6, 10, 12, 20, and 25) have not required systemic chemotherapy for more than 34 months since the start of CTL therapy.

Presence of LMP2-specific T cells in CTL product correlates with outcome

Twenty-five patients received CTL products containing T cells specific for the EBV antigen LMP2 expressed in NPCs. These

Table 1 Patient characteristics

ID	Age (years)/sex	PS	Site of disease	Primary ChemoRT[a]	Cycles of GC	CTL dose × 10^8 ([b])	Best overall response	OS (weeks)
1	49/M	0	PNS and cervical LN	Yes	4	10.4 (6)	PR	170.9
2	57/M	0	PNS and bone	Yes	4	7.4 (5)	PR	119.4
3	44/M	1	Bone and mediastinal LN	Yes	4	4.9 (3)	SD	32.0
4	63/F	0	Cervical LN, PNS, and axillary LN	Yes	4	4.4 (3)	PR	28.4
5	61/M	1	PNS, cervical LN, and liver	Yes	6	3.4 (2)	PR	80.3
6	64/M	0	Liver, lung, and mediastinal LN	Yes	4	9.7 (6)	SD	202.6[c]
7	35/M	1	Mediastinal LN, paraaortic LN, and lung	Yes	6	0.1 (1)	PR	207.3[c]
8	47/M	1	PNS and parotid	Yes	4	11.6 (6)	SD	204.6[c]
9	65/M	1	Liver, cervical LN, and lung	No	4	10.1 (6)	PR	130.1
10	57/M	1	Mediastinal LN, lung, and pleural	Yes	4	10.8 (6)	PR	190.1[c]
11	40/M	1	Liver, PNS, cervical LN, and abdominal LN	No	4	11.3 (6)	PR	108.3
12	43/M	0	PNS, cervical LN, and mediastinal LN	No	4	10.8 (6)	PR	196.0[c]
13	40/M	1	PNS, bone, and cervical LN	No	4	11.2 (6)	PR	149.4
14	69/M	1	Cervical LN	Yes	4	10.1 (6)	SD	83.0
15	50/F	0	Mediastinal LN and abdominal LN	Yes	4	9.6 (6)	PR	112.1
16	56/M	1	Pleural and LN (supradiaphragmatic)	Yes	4	8.0 (5)	CR	49.6
17	58/M	1	Mediastinal LN, cervical LN, liver, and bone	Yes	4	5.1 (3)	SD	42.1
18	57/M	0	Cervical LN	Yes	3	–	SD	14.3
19	45/F	1	Cervical LN, axillary LN, and chest wall	Yes	2	–	PD	28.7
20	54/F	1	Bone and lung	Yes	4	9.1 (6)	PR	173.4[c]
21	57/M	1	PNS, lung, and mediastinal LN	Yes	4	10.2 (6)	PR	134.1
22	55/F	1	PNS, cervical LN, lung, and bone	No	6	9.6 (6)	CR	131.4
23	63/M	0	PNS, cervical LN, and bone	Yes	4	10.8 (6)	PR	142.6
24	34/F	1	Bone, paravertebral, and pelvis	Yes	4	4.2 (3)	PR	43.7
25	63/M	0	Liver, cervical LN, and PNS	Yes	4	8.4 (6)	CR	175.6[c]
26	45/M	0	Liver and abdominal LN	Yes	4	10.2 (6)	PR	174.4[c]
27	25/F	1	Liver, abdominal LN, lung, and bone	Yes	4	2.8 (2)	PR	34.3
28	58/M	0	Lung	Yes	1	–	–	8.9
29	41/M	0	PNS and cervical LN	Yes	4	12.0 (6)	SD	167.6[c]
30	62/F	1	PNS	Yes	4	7.8 (6)	SD	172.4[c]
31	70/F	1	Lung, liver, and LN (hilar, paratracheal, right upper)	Yes	3	3.0 (2)	PR	31.3
32	69/M	1	Bone, PNS, cervical LN, and liver	No	4	10.2 (6)	PR	80.7
33	66/M	1	Liver, abdominal LN, and bone	Yes	4	3.7 (2)	SD	22.0
34	47/M	1	PNS, parotid, orbits, and axillary LN	Yes	4	9.4 (6)	PR	111.6
35	39/F	0	PNS and parotid	Yes	4	8.3 (6)	SD	161.4[c]
36	66/M	1	Bone, PNS, cervical LN, and lung	No	4	10.0 (6)	PR	159.9[c]
37	77/M	1	Liver and bone	Yes	4	9.0 (6)	PR	90.6
38	61/M	1	PNS	Yes	4	10.8 (6)	SD	97.7

CR, complete response; CTL, cytotoxic T lymphocyte; F, female; GC, gemcitabine/carboplatin; LN, lymph node; M, male; PD, progressive disease; PNS, paranasopharynx; PR, partial response; PS, performance status; RT, radiotherapy; SD, stable disease.
[a]Patients in this study may have received prior chemoradiation therapy as part of a primary curative modality treatment strategy for early stage NPC. Chemotherapy used in chemoradiation consisted of cisplatin with or without 5-flurouracil. [b]Number of CTL infusions written in parenthesis. [c]Alive at the time of analysis with their survival time censored at the time of last follow-up.

patients had a significantly improved OS compared with those (n = 9) who received CTL product lacking LMP2 specificity (hazard ratio: 0.35; 95% CI: 0.14–0.84; P = 0.014) (**Figure 3** and **Supplementary Table S2**). Survival analysis for LMP1 and EBNA1 was not performed due to the low number of positive CTL products.

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Table 2 Response and CBRs

	Chemotherapy phase[a] (n = 38)		Immunotherapy phase[b] (n = 35)		For combined chemoimmunotherapy (n = 35)	
	Number	Percentage	Number	Percentage	Number	Percentage
BOR						
CR	3	7.9	2	5.7	3	8.6
PR	21	55.3	13	37.1	22	62.9
SD	12	31.6	7	20.0	10	28.6
PD	1	2.6	11	31.4	0	–
Not assessed	1	2.6	2	5.7	0	
RR						
No of patients with BOR = CR/PR	24		15		25	
RR, % (95% CI)	63.2 (46.0–78.2)		42.9 (26.3–60.7)		71.4 (53.7–85.4)	
CBR						
No of patients with BOR = CR/PR/SD	36		22		35	
CBR, % (95% CI)	94.7 (82.3–99.4)		62.9 (44.9–78.5)		100.0 (90.0–100.0)[c]	

BOR, best overall response; CBR, clinical benefit rate; CI, confidence interval; CR, complete response; PD, progressive disease; PR, partial response; RR, response rate; SD, stable disease.
[a]Response to chemotherapy was determined by comparing pre- and post-chemotherapy imaging studies. [b]Response to immunotherapy was determined by comparing pre- and post-CTL imaging studies with the new tumor baseline established after the completion of chemotherapy. [c]One-sided 97.5% CI.

Table 3 OS of patients on GC-CTL, PGC-5-FU, and PGC clinical trials

	GC-CTL[a] (n = 35) [A] Patients who received CTL	GC-CTL[b] (n = 38) [B] All patients	PGC-5-FU[c] (n = 28) [C] All patients	PGC[d] (n = 32) [D] All patients
Follow-up (months)				
Median	29.9	26.6	21.4	17.7
Range	5.1–47.7	2.0–47.7	1.8–42.4	4.4–43.9
OS				
No. of events/patients	23/35	26/38	21/28[c]	26/32[d]
Median OS, months (95% CI))	29.9 (20.8–39.3)	26.6 (18.6–34.4)	21.4 (14.1–30.0)	18.3 (13.2–23.1)
1-year OS, % (95% CI)	77.1 (59.5–87.9)	71.1 (53.9–82.8)	75.0 (54.6–87.2)	81.3 (63.0–91.1)
2-year OS, % (95% CI)	62.9 (44.8–76.5)	57.9 (40.8–71.7)	42.9 (24.6–60.0)	29.5 (14.8–45.9)
3-year OS, % (95% CI)	37.1 (21.1–52.7)	34.2 (19.8–49.1)	25.0 (9.9–43.6)	16.4 (6.0–31.3)

5-FU, 5-flurouracil; C, carboplatin; CI, confidence interval; CTL, cytotoxic T lymphocyte; G, gemcitabine; P, paclitaxel.
[a]35 patients who received both CG and CTL "per-protocol". [b]38 patients analyzed as "intention-to-treat". [c]OS for the PCG + 5-FU trial are consistent with previously reported values.[5] [d]OS for the PCG trial is slightly different from previously reported values. Our results include additional follow-up data collected as at 2007 that contained 11 more deaths as compared with 15 deaths reported in the original publication.[6]

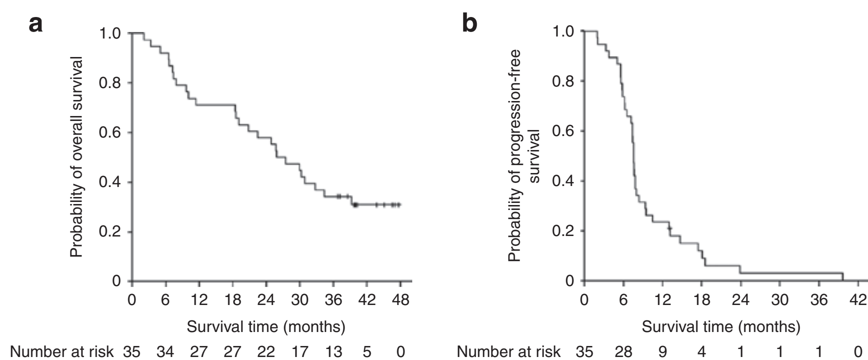

Figure 2 Survival outcomes for patients on study. (**a**) Overall survival (OS). (**b**) Progression free survival.

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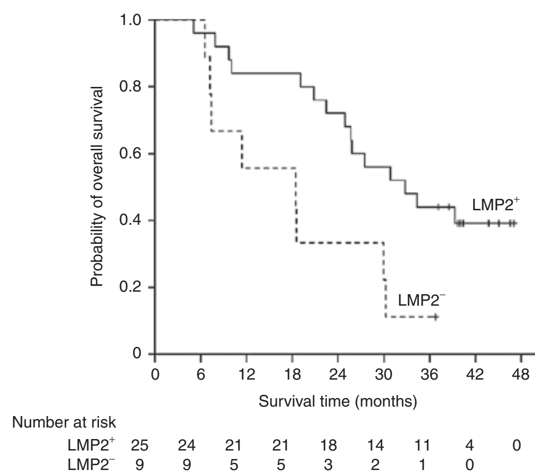

Figure 3 Presence of LMP2-specific T cells in cytotoxic T lymphocyte (CTL) product correlates with outcome. Patients (n = 25) receiving CTL products that contained LMP2-specific T cells had a significantly improved overall survival compared with those (n = 9) who received CTL product lacking LMP2 specificity (hazard ratio: 0.29; 95% CI: 0.12–0.74; P = 0.006). One patient was excluded from the analysis because we could not determine the LMP2 status of the infused CTL line.

Immune response after CTL infusion

We determined the frequency of EBV-specific T cells in the peripheral blood before chemotherapy and before each CTL infusion using autologous lymphoblastoid cell lines (LCLs) as stimulators in interferon-γ (IFN-γ) enzyme-linked immunospot (Elispot) assays. As a control, we measured the frequency of CMVpp65-specific T cells. There was no significant decline after chemotherapy or increase after CTL infusion of EBV-specific T cells; in addition, there was no change in endogenous, CMV-specific T-cell immunity (**Supplementary Figure S2A,S2B**). We also examined the precursor frequency of LMP2-specific T cells in patients who received CTL products with and without LMP2 specificity. Again, no significant changes were observed after CTL infusions for either group (**Supplementary Figure S2C,S2D**).

High EBV-DNA levels correlate with tumor burden and predict poor outcome

A total of 249 paired data points of EBV-DNA level and tumor diameter from 35 patients who received chemoimmunotherapy were analyzed. The EBV-DNA load correlated with tumor diameter (correlation coefficient: 0.696; 95% CI: 0.617–0.774; $P < 0.001$) above a threshold of an EBV-DNA load of ~150 copies/ml. Cox regression analysis revealed that for every twofold increase in EBV-DNA load at baseline, the mortality hazard increased by ~28% (hazard ratio: 1.28; P = 0.004). In contrast, no correlation was observed between baseline EBV-DNA levels and response to chemotherapy and/or CTL infusion.

Elevated levels of IP-10 and MIP-3α at baseline correlate with poor outcome

Plasma was isolated from patients at baseline and before each CTL infusion, and the levels of cytokine/chemokine were determined. Of the 27 cytokines/chemokines evaluated, only baseline IP-10 and MIP-3α levels were inversely associated with long-term survival (P = 0.029 and P = 0.035, respectively; **Supplementary Figure S4**).

Comparisons with PGC trials

Exploratory analysis was performed to compare survival outcomes between the GC-CTL trial and two independent first-line Paclitaxel–Gemcitabine–Carboplatin (PGC)-based chemotherapy trials performed at our center.[5,6] The first study was a single-arm phase 2 open-label study that enrolled 32 patients with locally recurrent or metastatic NPC who have not received previous palliative chemotherapy to a first-line chemotherapy regimen consisting of six cycles of PGC.[5] When published in 2005, the study reported the highest response rates and OS rates achieved for metastatic NPC at the time. A follow-up study several years later evaluated the benefit of maintenance of 5-fluorouracil (5-FU) chemotherapy added onto a back-bone of induction PGC in a similar cohort of NPC patients.[6] This regimen, although highly efficacious, was also highly toxic with almost 80% of patients experiencing grade 3 and 4 neutropenia.

Although nonrandomized, the patient characteristics across the GC-CTL and PGC trials were broadly similar (data not shown). Using individualized patient data, survival was analyzed on both an intention-to-treat and "per-protocol" basis, and these data are summarized in **Table 3**. Patients who received per-protocol GC-CTL had an improved median OS of 29.9 months, as compared with 17.7 months for PGC and 21.4 months for PGC-5-FU. Two-year OS rates were 62.9, 42.9, and 29.5% and 3-year OS rates were 37.1, 25, and 16.4% for the GC-CTL, PGC-5-FU, and PGC trials, respectively. These results support a potential therapeutic benefit for incorporating adoptive CTL therapy into a front-line chemotherapy strategy for advanced NPC.

DISCUSSION

In this study, we evaluated the feasibility and safety of administering chemotherapy in combination with EBV-CTLs in the first-line setting for patients with metastatic and/or recurrent NPC. We found that combining four cycles of GC followed by up to six cycles of EBV-CTLs (GC-CTL) is feasible, safe, and resulted in the highest median OS and long-term OS rates reported for this patient population.

Infusion of EBV-CTLs post GC was safe with no grade 3 or 4 adverse events. While the administration of GC resulted in fewer grade 3 or 4 nonhemtological toxicities (5.2%) in comparison with the published PGC (18%) and PGC-5-FU (30%) clinical trials at our center[5,6], we observed one treatment-related death (pneumonia), highlighting the need to minimize toxic therapies in this patient population.

In the intention-to-treat analysis, GC-CTL therapy (n = 38) resulted in a median OS of 26.6 months and 2-year OS rate of 57.9%. If the analysis was restricted to patients who received CTL (n = 35), the median OS increased to 29.9 months and the 2-year OS rate to 62.9%. Regardless of the performed analysis, the median OS and the 2-year OS rate were higher compared with patients receiving PGC-5-FU (21.4 months, 42.0%) or PGC (17.7 months, 29.5%; **Table 2**) on previous prospective first-line NPC trials conducted at our center.[5,6] Patients receiving GC-CTL also had improved outcomes in comparison with patients treated at other centers with

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chemotherapy alone. Siu *et al.*[17] reported a median OS of 14 months with a 2-year OS rate of 25% for 44 chemotherapy naive metastatic NPC patients who received doxorubicin, cisplatin, methotrexate, bleomycin, and cyclophosphamide. Two studies reported the outcomes of 86 metastatic NPC patients receiving gemcitabine and oxaliplatin or gemcitabine and cisplatin, a chemotherapy regimen which closely matches the one used in our study.[18,19] Both studies reported inferior median OS rates (15 and 19.6 months). Two-year OS rates were only reported for one of the studies, which was also lower (20%) in comparison with GC-CTL.[18]

As reported for other immunotherapy studies, we observed a discrepancy between a short PFS (7.6 months) and a much longer 2-year OS rate.[20] To determine whether the rate of tumor growth changed after CTL therapy, we measured the change in tumor size over time (**Supplementary Figure S2A**). Several patients had a decline of tumor growth after initial progression, and late tumor responses were observed in at least two patients, suggestive of delayed antitumor and/or immunomodulatory effects of infused CTLs. Importantly, five patients with distant metastases did not require further salvage chemotherapy for nearly 3 years after initiation of CTL.

While EBV-CTLs were infused after 4–6 cycles of chemotherapy, patients were not lymphopenic at the time of CTL infusion (data not shown). This most likely explains why we did not observe a significant *in vivo* expansion in the peripheral blood of adoptively transferred CTLs, a finding that was previously reported when CTLs were given as monotherapy to NPC patients.[13] Physiologically, however, lymphocytes traffic readily and the blood only contains 2% of the entire lymphocyte pool.[21] Thus, observing no expansion in the peripheral blood does not exclude expansion of EBV-CTLs in lymphoid organs and/or tumor sites. Nevertheless, the chemotherapy could have enhanced CTL activity by destroying immunosuppressive cells and/or inducing the expression of immunodominant EBV antigens within tumors.[22,23] Vaccination is another strategy to boost the expansion of adoptively transferred T cells *in vivo*.[24,25] In this regard, a recombinant vaccinia virus (MVA-EL) encoding an LMP2/EBNA1 fusion protein was shown to augment LMP2-/EBNA1-specific CD4- and CD8-positive T-cell responses in NPC patients,[26] paving the way to combine the adoptive transfer of EBV-CTL with vaccination in future clinical studies for patients with EBV-positive malignancies including NPC.

Clinical outcomes correlated with the presence of T cells specific for LMP2 in the CTL product, which is one of the three EBV antigens predominately expressed in NPC.[10] Only 25 out of the 34 infused CTL lines had LMP2 specificity, and our ability to generate CTL lines with LMP2 specificity correlated with the presence of LMP2-specific T cells in the peripheral blood at time of venesection (data not shown). We did not perform an outcome analysis of the other two EBV antigens predominantly expressed in NPC (LMP1 and EBNA1) due to the low number of positive CTL products. High-resolution human leukocyte antigen (HLA) typing was performed on all patients, and multiple correlative analyses between HLA type, specificity of CTL lines, and outcomes were performed. While no correlation was found, this result has to be considered preliminary due to our relatively small sample size and the diversity of HLA types.

CTL products for this clinical trial were generated by the "standard LCL methods",[13] which took 8–22 weeks, and relied on "life" EBV to generate autologous LCLs as antigen-presenting cells. Recently, the development of alternative methods to rapidly generate antigen-specific T cells, including T cells specific for EBNA1 and LMP2 using overlapping peptide libraries, has seen clinical efficacy for NPC in the salvage setting.[15,27,28] These methods generate CTL products with greater anti-NPC activity in a shorter time, facilitating the integration of CTL therapy into the current treatment armamentarium for NPC.

EBV-DNA, IP-10, and MIP-3α levels at study entry also correlated with OS. As previously reported in NPC patients treated with chemotherapy, high EBV-DNA levels and elevated MIP-3α levels correlated with poor outcome.[29–31] In contrast to other studies, we did not find poorer outcomes for patients with increased levels of IL-6 and IL-8.[31,32] Clearly, multicenter studies with larger cohorts of NPC patients are needed to identify predictive cytokines and chemokine patterns.

In summary, our study incorporates T-cell therapy into standard first-line treatment paradigms for NPC. Patients received a cytoreductive regimen of GC chemotherapy followed by the infusion of EBV-CTLs. GC-CTL therapy was well tolerated, and a subgroup of patients achieved prolonged disease stabilization and good performance status with no requirement for systemic treatment for ~3 years. With long-term follow-up, GC-CTL has demonstrated promising clinical activity in a relatively large and well-defined cohort of patients. This study reports one of the best survival outcomes to date for advanced stage NPC, the majority with distant and multiple sites of metastases, setting the stage for a future randomized study of chemotherapy with and without CTL therapy that is already underway.

MATERIALS AND METHODS

Study design. This prospective, open-label single-center nonrandomized phase 2 study was approved by the Institutional Review Board of the National Cancer Centre and the Health Sciences Authority, Singapore. The primary objective of this study was to determine the PFS for combined chemoimmunotherapy. Secondary objectives were the determination ofOS, PFS for CTL immunotherapy, response rate (RR), clinical benefit rate, toxicity, and treatment compliance. Response was evaluated by computer tomography (CT) or magnetic resonance imaging using Response Evaluation Criteria in Solid Tumors (RECIST). Patients with previously untreated histologically proven, EBV-associated metastatic or locally recurrent NPC (WHO type II/III) were eligible for this study. 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 >2× upper limit of normal value, or aspartate aminotransferase/alanine aminotransferase >3× upper limit of normal value were excluded. Patients with positive serology for human immunodeficiency virus were also excluded. All patients were treated between October 2008 and February 2011.

Generation of EBV-transformed LCLs and EBV-CTLs. Autologous LCLs and EBV-CTLs were generated according to current Good Manufacturing Practice guidelines as previously described.[13] After expansion, EBV-CTLs were tested for sterility, HLA identity, immunophenotype, and EBV specificity at the time of cryopreservation.

Chemotherapy and CTL therapy. The study design is summarized in **Figure 1**. Patients were venesected 300 ml of peripheral blood to generate LCL and EBV-CTL. Promptly thereafter, patients received chemotherapy

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consisting of gemcitabine (1,000 mg/m^2) and carboplatin (AUC 2) on days 1, 8, and 15 every 4 weeks for a total of four cycles. If necessary, patients received two additional cycles of chemotherapy to allow for extra time to generate sufficient CTLs for infusion. The relative dose intensity of chemotherapy (i.e., the ratio of the delivered dose intensity versus planned dose intensity) for chemotherapy was also calculated. Two to 4 weeks after the last course of chemotherapy, EBV-CTLs were administered at a dose of 1×10^8 cells/m^2 on weeks 0, 2, 8, 16, 24, and 32. Radiological imaging was performed at baseline, before the third cycle of chemotherapy, and before the first, third, fourth, fifth, and sixth cycle of CTL immunotherapy. New peripheral blood samples were obtained before commencement of chemotherapy and before each CTL infusion.

Plasma EBV-DNA analysis. Plasma samples were obtained from patients at baseline and before each CTL infusion. DNA was extracted from 800 μl of plasma using the QIAmp blood kit (Qiagen, Hilden, Germany), and EBV-DNA was amplified by real-time polymerase chain reaction (Applied Biosystems Fast PCR, Foster City, CA) as previously described.[33]

Elispot assay. The frequency of EBV-antigen-specific T cells was measured using IFN-γ Elispot assays as previously described.[13] Briefly, CTLs were stimulated with HLA-restricted peptides derived from EBV antigens (Genemed Synthesis, San Antonio, TX) or overlapping peptide mixes for BZLF1, BRLF1, BMRF1, EBNA1, EBNA3A, EBNA3B, EBNA3C, LMP1, and LMP2. Peptide mixes contained 15 amino-acid peptides covering the entire length of the corresponding protein with a 11 amino-acid overlap (pepmixes; JPT Peptide Technologies, Berlin, Germany). No peptide and pepmixes for the CMV antigen pp65 (CMVpp65) served as negative control. The frequency of EBV-, LMP2-, and CMVpp65-specific T cells in the peripheral blood of patients at baseline and before each CTL infusion were determined by using autologous LCL or LMP2 and CMVpp65 pepmixes. Briefly, antigen-specific T cells were reactivated with LCLs or pepmixes and after 8–10 days an Elispot assay was performed. Developed Elispots were analyzed by ZellNet Consulting (New York, NY). Spot-forming cells were calculated and expressed as spot-forming cells per 10^5 cells.

Flow cytometry. Monoclonal antibodies were obtained from Becton Dickinson (San Jose, CA) and are listed in the appendix. Flow cytometric analysis was performed using a FACSAria I instrument and FACSDiVa software (Becton Dickinson). The monoclonal antibodies included anti-CD3, -CD4, -CD8, -CD16, -CD19, -CD27, -CD28, -CCR7, -CD62L, -CD45RA, -CD56, -TCRαβ, and -TCRγδ.

Serum cytokine/chemokine analysis. A 27-plex human cytokine/chemokine luminex multiplex bead array assay kit (Invitrogen; Carlsbad, CA) was used to measure the following cytokines: IL-3, IL-4, IL-6, IL-8, IL-9, IL-10, IL-15, IL-17, IL-21, (CXCL10) IP-10, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL20 (MIP-3sα), MCP-1, IFN-α2, IFN-γ, EGF, FGF-2, VEGF, TGFA, CD40L, fractalkine, GM-CSF, G-CSF, GRO, MDC, and eotaxin. Each undiluted plasma sample was assayed in duplicate according to the protocol provided by the manufacturer.

Statistical analysis. Response and clinical benefit rates were summarized as frequency and percentage with corresponding 95% CI estimated based on the exact method.

Overall PFS was calculated from the date of entry to trial to date of disease progression or death from any causes, whichever occurs first, and PFS for the CTL immunotherapy phase was calculated from the date of tumor evaluation before initiation of immunotherapy to date of disease progression or death from any causes, whichever occurred first. OS was measured from the date of study entry to date of death from any causes. Patients who did not develop any of these time-to-event end points were censored at the date of last follow-up.

The Kaplan–Meier method was used to estimate survival distribution, and the log-rank test was used to compare differences between survival curves. Cox proportional hazard model was fitted to estimate hazard ratio to assess the association of the LMP2 status with each survival end point, and a two-sided P value <0.05 was considered statistically significant. Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

For EBV-DNA analysis, each blood sample was matched to computer tomography scan tumor diameter measurements nearest to the blood sample collection date, and tumor diameter and EBV copy number were both log transformed (base e) due to positive skewness. Because the log of 0 is undefined, an EBV value below a detection limit was treated as EBV = 1 and note that log (1) = 0. Pearson's correlation coefficient for log tumor diameter and log EBV was estimated, with bootstrap method (200 replicates) used to allow clustering of multiple data points within each patient. Fractional and natural polynomials were used to explore nonlinear relationships in regression analysis, and the mixed model was used to estimate the relation between log tumor load and log EBV. Cox regression was used to assess baseline log EBV and survival time.

Plasma cytokine concentrations were obtained using the Luminex platform for patients at each phase of the study. The concentration values in pg/ml were log10 transformed to approximate normality. The log10 transformed concentration values were then used in a two-way ANOVA with the study phase as one of the factors and survival at 1 year, survival at 2 years, or long-term survivor status (survivors with no chemotherapy after immunotherapy) as the other factor. An interaction term was included in the ANOVA. The data for the ANOVA tests on the Luminex concentrations were processed using Accelrys Pipeline Pilot, and statistical analysis was performed in R version 2.12.2. All other analyses were performed in SAS version 9.3 (SAS Institute, Cary, NC).

SUPPLEMENTAL MATERIAL

Figure S1. Characteristics of generated EBV-CTL lines.
Figure S2. Precursor frequency of EBV-, LMP2-, and CMVpp65-specific T cells post GC-CTL.
Figure S3. Tumor growth post GC-CTL therapy.
Figure S4. Plasma IP-10 and MIP3α correlate with OS.
Table S1. Toxicities associated with chemotherapy and CTL infusion.
Table S2. Overall survival of patients by LMP2 status.

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