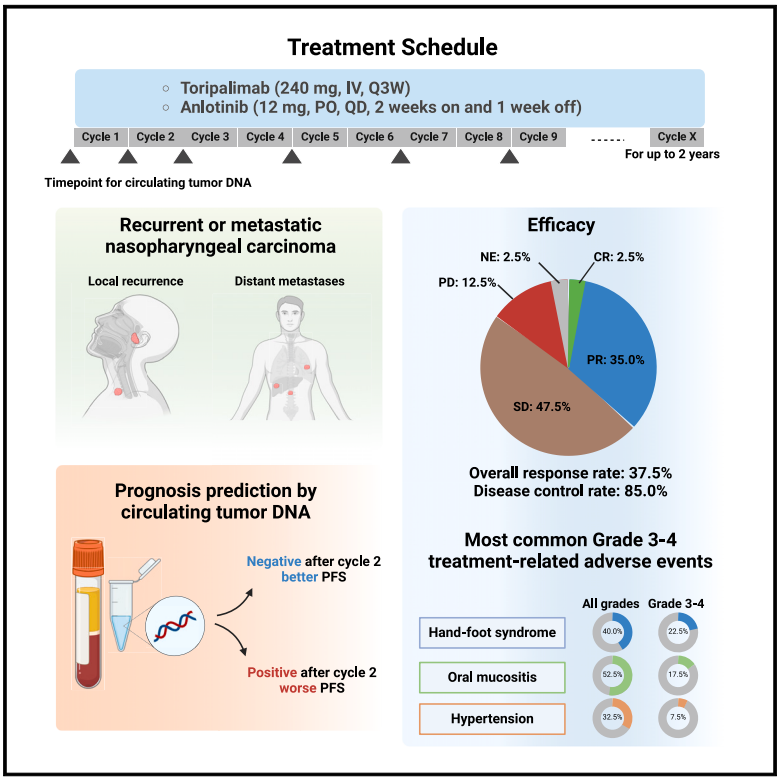
Cell Reports
Medicine

Article

Toripalimab plus anlotinib in patients with recurrent or metastatic nasopharyngeal carcinoma: A multicenter, single-arm phase 2 trial (TORAL)

Graphical abstract



Highlights

- Toripalimab plus anlotinib shows promising efficacy in RM-NPC
- The regimen is well tolerated with an acceptable safety profile
- Plasma ctDNA is associated with treatment response and survival outcome
- Dynamic change of ctDNA may be a potential predictive marker for disease progression

Authors

Yuchen Zhang, Qihua Zou, Baitian Zhao, ..., Yingxian Liu, Yi Xia, Qingqing Cai

Correspondence

xiayi@sysucc.org.cn (Y.X.), caiqq@sysucc.org.cn (Q.C.)

In brief

Zhang et al. report the promising efficacy and acceptable safety profile of toripalimab plus anlotinib for patients with recurrent or metastatic nasopharyngeal carcinoma and demonstrate the potential predictive role of plasma circulating tumor DNA.

Zhang et al., 2024, Cell Reports Medicine 5, 101833
December 17, 2024
https://doi.org/10.1016/j.xcrm.2024.101833

CellPress

Cell Reports Medicine

CellPress
OPEN ACCESS

Article

Toripalimab plus anlotinib in patients with recurrent or metastatic nasopharyngeal carcinoma: A multicenter, single-arm phase 2 trial (TORAL)

Yuchen Zhang,[1,2,7] Qihua Zou,[1,2,7] Baitian Zhao,[1,3,7] Ning Su,[4] Zhihua Li,[5] Xicheng Wang,[6] Panpan Liu,[1,2] Xiaopeng Tian,[1,2] Xiaojie Fang,[1,2] Jun Cai,[1,3] Lirong Li,[1,2] Yingxian Liu,[1,2] Yi Xia,[1,2,*] and Qingqing Cai[1,2,8,*]

[1]State Key Laboratory of Oncology in South China, Guangdong Key Laboratory of Nasopharyngeal Carcinoma Diagnosis and Therapy, Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center, Guangzhou 510060, P.R. China
[2]Department of Medical Oncology, Sun Yat-sen University Cancer Center, Guangzhou 510060, P.R. China
[3]Department of Clinical Research, Sun Yat-sen University Cancer Center, Guangzhou, P.R. China
[4]Department of Oncology, Guangzhou Chest Hospital, Guangzhou 510095, P.R. China
[5]Department of Oncology, Sun Yat-sen Memorial Hospital, Guangzhou, Guangdong, P.R. China
[6]Department of Oncology, The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangdong Pharmaceutical University, Guangzhou 510062, P.R. China
[7]These authors contributed equally
[8]Lead contact
*Correspondence: xiayi@sysucc.org.cn (Y.X.), caiqq@sysucc.org.cn (Q.C.)
https://doi.org/10.1016/j.xcrm.2024.101833

SUMMARY

Treatment options for patients with recurrent or metastatic nasopharyngeal carcinoma (RM-NPC) after failure of platinum-based therapy are limited. In this phase 2 trial, 40 patients with RM-NPC who failed platinum-based chemotherapy receive toripalimab plus anlotinib regimen. The objective response rate is 37.5%, and the disease control rate is 85.0%. With a median follow-up of 17.4 months, the median progression-free survival (PFS) is 9.5 months and 1-year overall survival rate is 73.3%. The most common treatment-related grade 3–4 adverse events are hand-foot syndrome (22.5%) and oral mucositis (17.5%). Analyses of plasma circulating tumor DNA (ctDNA) demonstrate that the blood tumor mutation burden at cycle 1/2 is associated with response and PFS, and disease progression indicated by ctDNA precedes radiological progression by a median of 2.3 months. In conclusion, toripalimab plus anlotinib is well tolerated and shows promising efficacy in patients with RM-NPC, and ctDNA could be a potential predictive biomarker. The trial is registered at ClinicalTrials.gov (NCT04996758).

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a distinct subtype of head and neck cancers, with the highest prevalence in Southeast Asia and South China.[1] Approximately 20% of patients with NPC develop recurrent or metastatic disease and the prognosis remains poor, with a median overall survival (OS) less than 20 months[2–4] The standard first-line therapy for recurrent or metastatic NPC (RM-NPC) is gemcitabine plus cisplatin, and the efficacy was further improved with the addition of programmed death-1 (PD-1) inhibitors.[5–7] Nevertheless, there is no standard treatment option for patients who are refractory to or relapsed after first-line platinum-based therapy.

PD-1 blockade is an effective choice for subsequent-line treatment of RM-NPC, with an overall response rate (ORR) from 20.5% to 34.1% and acceptable safety profiles.[8–11] Toripalimab, a humanized IgG4 monoclonal antibody targeting PD-1, was approved in 2021 in China, and subsequently by the USA Food and Drug Administration and the European Medicines Agency for the treatment of RM-NPC.[6,11] In consideration of its durable response and mild toxicity, the combination of PD-1 inhibitors with other therapeutic agents might be the novel treatment strategies.

Several clinical trials have shown that angiogenesis was a potential therapeutic target for NPC.[12–14] Anlotinib was a tyrosine kinase inhibitor (TKI) that targets vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor, and platelet-derived growth factor receptors.[15,16] Our team reported the promising antitumor effect of anlotinib for RM-NPC (ORR, 20.5%; disease control rate [DCR], 71.8%) in the third-line settings and beyond.[17] Recently, the combination of anti-VEGFR TKIs or anti-VEGF antibodies with PD-1 inhibitors yielded an encouraging efficacy for RM-NPC, with an ORR of 55%–65% and 33%–34% in patients with and without prior PD-1 inhibitor treatment, respectively.[18–20] However, dynamic biomarkers that provide information in predicting clinical outcomes and risk of disease progression have not been well established for this new strategy.

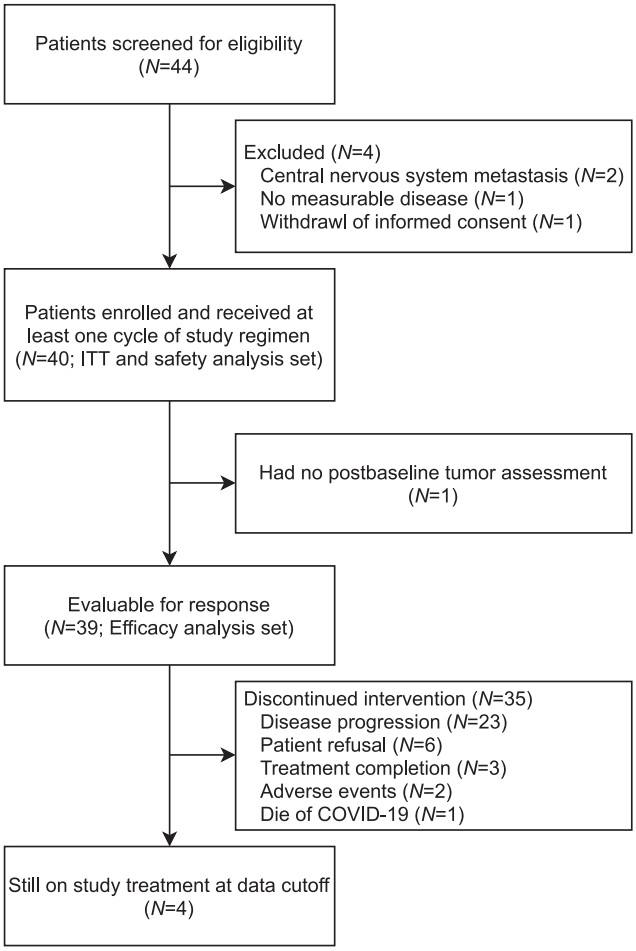


Figure 1. Flow diagram

In this phase 2 study (TORAL), we evaluated the efficacy and safety profile of toripalimab plus anlotinib in patients with RM-NPC after failure of at least one line of platinum-based therapy, as well as the value of circulating tumor DNA (ctDNA) as a predictive biomarker.

RESULTS

Between October 30, 2021 and July 5, 2023, 44 patients with RM-NPC were screened, of whom 40 patients were enrolled and received at least one cycle of toripalimab plus anlotinib, which constituted the intention-to-treat (ITT) population (full analysis set) and safety analysis set (Figure 1). One patient refused further treatment due to grade 2 fatigue and thus had no postbaseline imaging assessment. The remaining 39 patients who had at least one postbaseline tumor assessment constituted the efficacy analysis population. Thirty-three patients who received at least two cycles of treatment and complied with the trial protocol constituted the per-protocol set (PPS).

The median age was 46 years (interquartile range [IQR], 39–54), and the proportion of male patients was 85.0%. Twenty-seven patients (67.5%) had both local-regional recurrence and distant metastases, 11 patients (27.5%) had distant metastases only, and 2 patients (5.0%) had local-regional recurrence only. Eighteen patients (45.0%) had at least two previous lines of therapy. More than half of the patients (23/40, 57.5%) had received immune checkpoint inhibitor (ICI) treatment before study entry, with a median duration from the last ICI treatment to the initiation of study regimen at 2.9 months (range, 1.0–27.1). Baseline characteristics are summarized in Table 1.

As of March 25, 2024, 4 patients remained in study treatment. The trial intervention was terminated in the remaining 35 patients for the following reasons: disease progression (N = 23), patient refusal (N = 6), treatment completion (N = 3), unacceptable toxicities (N = 2), and death due to COVID-19 (N = 1).

Efficacy

The median treatment cycle was 9 (range, 2–32). Among the 40 patients in the ITT population, the ORR was 37.5% (95% confidence interval [CI], 22.7–54.2) and DCR was 85.0% (95% CI, 70.2–94.3) (Table 2). Representative imaging features in patients with different responses are shown in Figures S1 and S2. In the efficacy analysis set, 29 patients (74.4%) had a decrease in target lesions from baseline (Figure 2A). The median time to first response was 1.5 months (IQR, 1.4–3.0 months), and the median time to confirmed response was 3.5 months (IQR, 2.6–9.6 months) (Figure 2B). In PPS, the ORR and DCR were 39.4% and 84.8%, respectively.

In post hoc response analyses of the efficacy analysis population, the ORR was 31.8% (7/22) vs. 47.1% (8/17) in patients with vs. without prior ICI exposure. The ORR was similar between patients with <3 vs. ≥ 3 months of immunotherapy-free period (27.3%, 3/11 vs. 36.4%, 4/11). However, progressive disease (PD) was observed in 3 patients with <3 months of immunotherapy-free period but not in any patients with >3 months of immunotherapy-free period. In comparison to patients with ≥2 lines of previous therapy, those with 1 line of previous therapy had higher ORR (52.4%, 11/21 vs. 22.2%, 4/18). Based on sites of recurrence, the ORR was 100.0% (2/2) in patients with local recurrence only, 36.4% (4/11) with distant metastasis only, and 34.6% (9/26) with both local recurrence and distant metastasis.

Similar ORR was achieved between different baseline Epstein-Barr virus (EBV)-DNA levels (EBV-DNA <10,000 copies/mL, 36.4% [8/22] vs. ≥ 10,000 copies/mL, 41.2% [7/17]). The best ORR was 60.0% (12/20) vs. 13.3% (2/15) in patients with ≥50% vs. <50% reduction in plasma EBV-DNA from baseline (C0) to cycle 1 (C1) (Table S1). In an analysis that excluded the 5 patients with PD after cycle 2 (C2), the best ORR was 52.2% (12/23) vs. 25.0% (2/8) in patients with ≥50% decrease vs. <50% decrease (Table S2).

With the median follow-up of 17.4 months (range, 1.7–29.8), the median progression-free survival (PFS) was 9.5 months (95% CI, 7.1–11.9) and the 1-year PFS rate was 38.0% (Figure 3A). The median OS was not reached, with the 1-year OS rate of 73.3% (Figure 3B). The median duration of response was 8.4 months (95% CI, 2.3–14.6) (Figure S3).

Safety

All enrolled patients experienced at least one treatment-related adverse event (TRAE). The TRAEs with a rate ≥50% included

Table 1. Baseline demographics and disease characteristics

Characteristics	Patients (n = 40)
Age	
Median (IQR), year	46 (39–54)
Gender	
Male	34 (85.0%)
Female	6 (15.0%)
ECOG performance status	
0	12 (30.0%)
1	27 (67.5%)
2	1 (2.5%)
Sites of recurrence	
Local-regional recurrence	2 (5.0%)
Distant metastases	11 (27.5%)
Local-regional recurrence with distant metastases	27 (67.5%)
Metastases sites	
Lung	21 (52.5%)
Liver	16 (40.0%)
Bone	21 (52.5%)
Distant lymph nodes	30 (75.0%)
Others[a]	6 (15.0%)
Previous lines of therapy for advanced disease	
1[b]	22 (55.0%)
2	14 (35.0%)
3	4 (10.0%)
Previous local-regional radiotherapy	
Yes	28 (70.0%)
No	12 (30.0%)
Previous ICI exposure	
Yes[c]	23 (57.5%)
No	17 (42.5%)
Median (range) duration from last ICI to study initiation, months	2.9 (1.0–27.1)
EBV-DNA	
Median (IQR), copies/mL	4,700 (850–155,250)
≥10,000	18 (45.0%)
<10,000	22 (55.0%)

Data are shown as number (%) unless otherwise specified.
[a]Others included subcutaneous (n = 1), pleura (n = 3), kidney (n = 1), adrenal gland (n = 1), and gum (n = 1) metastasis.
[b]Disease progression occurred in 5 patients within 6 months after radical chemo-radiotherapy for non-metastatic disease.
[c]Among the 23 patients, 21 patients used PD-1 inhibitors, one patient used PD-L1 inhibitors, and one patient used both PD-1 and PD-L1 inhibitors. EBV, Epstein-Barr virus; ECOG-PS, Eastern Cooperative Oncology Group performance status; ICI, immune checkpoint inhibitor; PD-1, programmed death-1; PD-L1, programmed death ligand-1; ULN, upper limit of normal.

Table 2. Antitumor activity

Response evaluation	Intention-to-treat population (n = 40)	Efficacy analysis population (n = 39)
Objective response rate, n (%)	15 (37.5)	15 (38.5)
95% CI	22.7–54.2	23.4–55.4
Disease control rate, n (%)	34 (85.0)	34 (87.2)
95% CI	70.2–94.3	72.6–95.7
Best overall response, n (%, 95% CI)		
Complete response	1 (2.5, 0.1–13.2)	1 (2.6, 0.1–13.5)
Partial response	14 (35.0, 20.6–51.7)	14 (35.9, 21.2–52.8)
Stable disease	19 (47.5, 31.5–63.9)	19 (48.7, 32.4–65.2)
Progressive disease	5 (12.5, 4.2–26.8)	5 (12.8, 4.3–27.4)
Not evaluable	1 (2.5, 0.1–13.2)	–

Data are shown as number (%). Responses were evaluated and confirmed according to RECIST version 1.1.

triglyceride elevation (N = 22, 55.0%), oral mucositis (N = 21, 52.5%), and proteinuria (N = 20, 50.0%) (Table 3). Twenty-three patients (57.5%) had grade 3 TRAEs, and the most common TRAEs included hand-foot syndrome (N = 9, 22.5%), oral mucositis (N = 7, 17.5%), and hypertension (N = 3, 7.5%). No grade 4 TRAEs were observed, and no treatment-related death occurred.

Sixteen patients (40.0%) required anlotinib dose reduction (to 10 mg in 12 patients and 8 mg in 4 patients). The most common reasons for dose reduction were hand-foot syndrome (N = 8) and oral mucositis (N = 6) (Table S3). Twenty-three patients (57.5%) required one or more anlotinib treatment deferral, and the most common reasons were hand-foot syndrome (N = 7), oral mucositis (N = 5), and abnormal transaminase (N = 3) (Table S4). Two patients discontinued trial intervention before disease progression due to TRAEs: one grade 3 pharyngolaryngeal pain, and one recurrent grade 3 lung infection with dyspnea.

ctDNA

For the measurement of ctDNA, peripheral blood samples were collected before treatment initiation/C0, at the end of C1 and C2, and thereafter every 2 cycles until disease progression (Figure S4).

At baseline, the most frequently mutated genes included *TP53* (26.3%), *CYLD* (18.4%), *NFKBIA* (18.4%), and *PIK3CA* (10.5%) (Figure 4A). Patients with mutation of *TP53* showed an inferior PFS, while no differences were observed in other high-frequency mutated genes (Figure S5). At baseline, the median blood tumor mutation burden (bTMB) was 4.1 muts/Mb (IQR, 1.0–9.6). Tumor tissue sample was available in only 8 cases (6 at the initial diagnosis and 2 at the time of trial enrollment). The analysis showed higher tumor mutation burden in ctDNA than in tissue samples (5.1 vs. 3.1 muts/Mb). Fourteen out of the 35 genetic alternations (40.0%) detected in the 8 cases with available tumor tissues were also detected in ctDNA (Figure 4B and Table S5).

A higher bTMB level at baseline was observed in patients with ≥4 recurrent sites (vs. 0–2), liver metastases (vs. none), bone metastases (vs. none), and EBV-DNA ≥10,000 copies/mL (vs. <10,000) (Figure S6). No significant differences were observed in patients with distant metastases only (vs. both distant

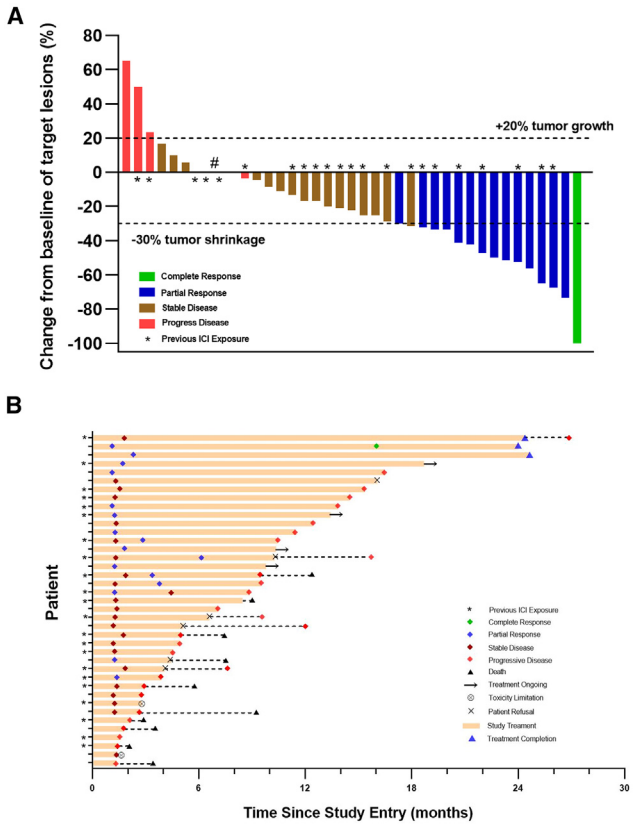


Figure 2. Tumor responses in patients from efficacy analysis population

(A) Best percentage change from baseline in target lesions according to RECIST version 1.1.

(B) Swimmer plot. #Four patients had a best percentage change of 0%, with three patients achieving stable disease and one patient achieving progressive disease (obvious progression of non-target lesions).

metastasis and local recurrence) or lung metastases (vs. none). In comparison to patients who achieved complete response, partial response, or stable disease (SD), patients with PD had comparable bTMB at C0 but significantly higher bTMB at C1 (Figure S7).

When dividing the patients into C0-negative or positive group, a similar response rate was observed (ORR: negative, 42.9% [3/7] vs. positive, 36.7% [11/30], $p = 1.000$); however, all five patients with PD were from the positive group (Table S6). Interestingly, C1-negative patients showed a trend for a higher ORR (negative, 57.1% [8/14] vs. positive, 36.7% [6/21], $p = 0.159$) (Table S7). Blood sample was available in 3 out of the 5 patients with PD after C1: two with bTMB increase (from 6.2 to 10.3 muts/Mb, and from 9.3 to 17.5 muts/Mb) and the remaining one with stable bTMB (from 16.5 to 14.4 muts/Mb). Comparison of the mutated genes after C1 vs. baseline showed that the percentages of the overlapping mutated genes were 100.0% (13/13), 91.7% (11/12), and 73.7% (14/19), respectively, of detected mutated genes after C1 (Table S8). C2 ctDNA status showed significant relevance with best response to the study regimen (negative, 73.3% [11/15] vs. positive, 18.8% [3/16], $p = 0.004$) (Figure 4C). For the four patients with the best response of SD in the ctDNA-negative group, 3 out of the 4 patients who manifested SD in the ctDNA-negative group were ctDNA negative at C0, and the remaining 1 patient had ctDNA clearance at C1. The best decrease in target lesions ranged from 0% to 25%. The PFS (range: 9.7 to 27.0 months) was longer than the median PFS of the entire cohort. In contrast, the median PFS in the 13 patients with SD in the ctDNA-positive group was 5.0 months (range: 2.7 to 12.4 months). The alluvial plots showed the dynamic change of ctDNA and its relationship with best response and EBV-DNA clearance (Figure S8). Stacked bar plots showed the relationship of ctDNA status with best response (Figure S9).

Subgroup analyses of ctDNA-positive rate at the single time points as well as the ctDNA clearance status from C0 to C1/C2 were performed to explore the differences between patients with and without prior ICI exposure. At C0, C1, and C2, the ctDNA-positive rate was similar between the two groups, and both groups showed the trend of decline (Table S9). Proportion of patients with cleared ctDNA was slightly higher in patients with no prior ICI exposure (Table S9).

In survival analyses, a better PFS was observed in the seven patients with negative C0 ctDNA (hazard ratio [HR] = 0.33; 95% CI, 0.12–0.96; $p = 0.032$) and C1 ctDNA (HR = 0.26; 95% CI, 0.11–0.61; $p = 0.001$) (Figure S10) according to bTMB. Similarly, after excluding the patients with PD at first postbaseline tumor assessment, patients with negative ctDNA at C2 also presented a superior PFS (HR = 0.07; 95% CI, 0.02–0.26; $p < 0.001$) (Figure 4D).

Furthermore, we would like to explore if there is any PFS difference between patients with sustained negative ctDNA (no detectable ctDNA at both C0 and C1/C2) and cleared ctDNA (change of bTMB from positive at C0 to negative at C1/C2). The results showed that no PFS differences were observed between these two groups at either C1 or C2, while both of the groups presented better PFS than the ctDNA uncleared group (Figure S11).

The percentage of patients with $\geq$50% EBV-DNA copy-number reduction was 85.7% (6/7) vs. 47.6% (10/21) in patients with vs. without ctDNA clearance at the end of first treatment cycle (Table S10). The percentage of patients with $\geq$50% EBV-DNA copy-number reduction was also higher in patients with (100% [9/9]) vs. without (56.3% [9/16]) ctDNA clearance at the end of second treatment cycle.

Dynamic monitoring of disease progression by ctDNA

During the treatment, longitudinal monitoring of ctDNA throughout the treatment period was performed. Among the 19 patients who achieved at least an SD and later experienced disease progression, the "significant increase" of bTMB was observed in 15 of the 19 patients, and the progression indicated by ctDNA preceded radiological progression by a median of 2.33 months (IQR, 1.47–4.40) (Figure 4E). In those 15 patients, *CYLD* (33.3%), *NFKBIA* (26.7%), and *TP53* (26.7%) were the most frequently mutated genes when a "significant increase" in bTMB was observed. The list of mutated genes at "significant increase" and their existence at baseline are presented in Table S8.

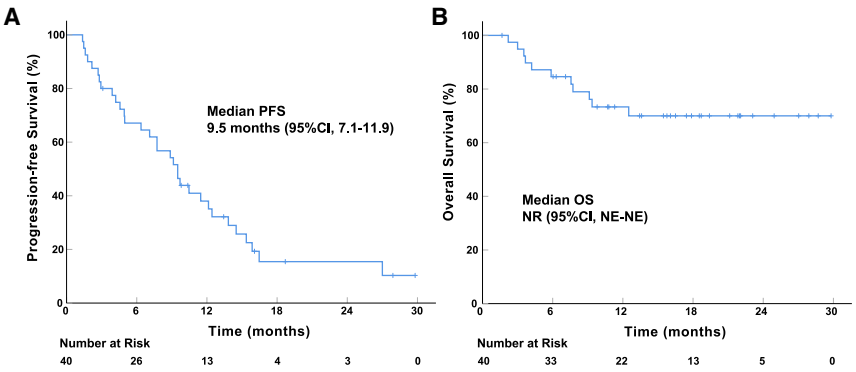


Figure 3. Kaplan-Meier estimate of survival in ITT population

(A) Progression-free survival.

(B) Overall survival. ITT, intention to treat.

DISCUSSION

This trial revealed a promising antitumor activity and an acceptable safety profile of this combined regimen. Exploratory analysis identified plasma ctDNA as a potential biomarker for response, prognosis prediction, and surveillance of disease progression.

For patients with RM-NPC who failed platinum-based systemic chemotherapy, the subsequent treatment options are limited, including chemotherapy and PD-1 inhibitors. A recent comparative phase 3 KEYNOTE-122 trial reported a similar ORR (21.4% vs. 23.3%) of pembrolizumab monotherapy and single-agent chemotherapy for platinum-pretreated RM-NPC.[21] Moreover, pembrolizumab showed a significantly lower incidence of TRAEs than chemotherapeutic agents.[21] Given the promising efficacy and mild toxicities, the strategy of PD-1-based combined therapy may have a good application prospect in the treatment of RM-NPC.

Antiangiogenic agents could effectively normalize tumor vascular network, alleviate the hypoxic status within the tumor microenvironment, and modulate immune cell populations, underlying the therapeutic potential of antiangiogenic agents in cancer immunotherapy.[22–24] The ORR with toripalimab plus anlotinib in this trial (47.1% and 31.8% in patients with and without ICI exposure, respectively) was similar to that reported previously for other combinations of PD-1 inhibitor plus antiangiogenic agents for RM-NPC. An ORR of 65.5% has been reported for camrelizumab plus apatinib, but this trial enrolled patients with no prior ICI exposure, and the proportions of local-regional recurrence only and one previous line of therapy were higher than those in the current study.[18] The same team reported camrelizumab plus famitinib in patients with RM-NPC previously treated with PD-1 blockade, and showed similar efficacy data to our study regimen.[19] Moreover, the combination of bevacizumab and sintilimab yielded an ORR of 54.5% in patients with ICI-naive, metastatic NPC who failed platinum-based chemotherapy.[20] However, these comparisons need to be interpreted with caution due to discrepancies between studies.

In comparison to previous studies of anlotinib plus PD-1 inhibitors for other solid tumors,[25–28] the toxicity profile of toripalimab plus anlotinib regimen in this trial was generally similar. Among the TRAEs, hand-foot syndrome (palmar-plantar erythrodysesthesia syndrome), oral mucositis (dental ulcer), hypertension, hypothyroidism, and alanine aminotransferase/aspartate aminotransferase elevation warrant further attention. It should be noted that a relatively higher rate of triglyceride/cholesterol elevation and proteinuria were observed in our study, but were manageable by supportive treatment. The difference might be partly explained by different tumor types and PD-1 inhibitors used. Furthermore, compared to other antiangiogenic TKIs in combination with PD-1 inhibitors for RM-NPC, there are also differences in toxicity profiles. Apatinib showed a higher incidence of hypertension, headache, and pharyngolaryngeal pain,[18] while famitinib was associated with significant hematological toxicities.[19] In contrast, anlotinib presented higher incidence of skin and mucosal toxicity (oral mucositis and hand-foot syndrome), leading to most of the dose reduction and treatment deferral. However, considering that the evaluation of symptomatic adverse events is characterized by a certain level of subjectivity, further comparative trials are needed.

Furthermore, we explored the value of ctDNA in predicting clinical outcomes by analyzing bTMB using a commercial gene panel targeting 437 cancer-related genes. Several studies reported the promising role of ctDNA in predicting clinical outcomes in patients undergoing a variety of treatments for various types of malignancies.[29–31] Recently, You et al. reported that the change of ctDNA from C0 to C2 was related to response and survival for patients with RM-NPC who received the gemcitabine, apatinib, and toripalimab regimen.[32] Han et al. showed that ctDNA could be used as a potential predictor for response to PD-L1 inhibitor plus anlotinib regimen and survival outcomes.[33] In the current study, bTMB level at C1 and C2 correlated closely to treatment response and PFS. Interestingly, for the patients with the best response of SD, patients with negative ctDNA at C2 had relatively better PFS than those with positive ctDNA. It indicated that the patients with negative ctDNA at C2 tended to have a more durable disease control period, including those with the best response of SD. Further validations are needed to verify this finding. It suggested that ctDNA might be a potential biomarker to predict progression, which needs to be further verified.

In the current study, we found that *CYLD* and *NFKBIA* were frequently mutated when patients presented a "significant increase" of bTMB, which might be an indicator of disease progression. Several studies have identified multiple mutations of negative regulators of nuclear factor κB (NF-κB) pathway, such as *CYLD*, *TRAF3*, and *NFKBIA*, in tumor samples of patients with NPC.[34–36] Functionally, the alternation of *CYLD* and *NFKBIA* plays an important role in NPC cell growth, apoptosis, as well as EBV replication.[34,35,37,38] Clinically, high NF-κB level was an independent negative prognostic factor for relapse-free survival in patients with NPC.[36] Targeting key players in the

Table 3. Treatment-related adverse events (*n* = 40)

TRAE	All grades	Grade 1–2	Grade 3
Triglyceride elevation	22 (55.0%)	20 (50.0%)	2 (5.0%)
Oral mucositis	21 (52.5%)	14 (35.0%)	7 (17.5%)
Proteinuria	20 (50.0%)	19 (47.5%)	1 (2.5%)
Hypothyroidism	19 (47.5%)	19 (47.5%)	0
Cholesterol elevation	17 (42.5%)	16 (40.0%)	1 (2.5%)
Hand-foot syndrome	16 (40.0%)	7 (17.5%)	9 (22.5%)
Hypertension	13 (32.5%)	10 (25.0%)	3 (7.5%)
AST elevation	13 (32.5%)	13 (32.5%)	0
Hypoalbuminemia	13 (32.5%)	13 (32.5%)	0
Bleeding[a]	12 (30.0%)	12 (30.0%)	0
ALT elevation	12 (30.0%)	12 (30.0%)	0
Fatigue	12 (30.0%)	12 (30.0%)	0
Cough	11 (27.5%)	11 (27.5%)	0
Leukopenia	11 (27.5%)	11 (27.5%)	0
Pharyngolaryngeal pain	10 (25.0%)	9 (22.5%)	1 (2.5%)
Musculoskeletal/joint pain	8 (20.0%)	7 (17.5%)	1 (2.5%)
Peripheral neuropathy	8 (20.0%)	8 (20.0%)	0
Neutropenia	8 (20.0%)	8 (20.0%)	0
Creatinine elevation	8 (20.0%)	8 (20.0%)	0
Anorexia	7 (17.5%)	6 (15.0%)	1 (2.5%)
Diarrhea	6 (15.0%)	6 (15.0%)	0
Hyperglycemia	6 (15.0%)	5 (12.5%)	1 (2.5%)
Rash	6 (15.0%)	6 (15.0%)	0
Abdominal pain	4 (10.0%)	4 (10.0%)	0
Anemia	3 (7.5%)	3 (7.5%)	0
Constipation	3 (7.5%)	3 (7.5%)	0
Nausea or vomiting	2 (5.0%)	2 (5.0%)	0
Lung infection	2 (5.0%)	1 (2.5%)	1 (2.5%)
Hoarseness	2 (5.0%)	1 (2.5%)	1 (2.5%)
Thrombocytopenia	1 (2.5%)	1 (2.5%)	0

Data are shown as number (%). ALT, alanine aminotransferase; AST, aspartate aminotransferase; TRAE, treatment-related adverse event.
[a]Sites of grade 1–2 bleeding included nasopharynx (*n* = 7), oropharynx (*n* = 3), intestinal tract (*n* = 1), and gum (*n* = 1).

NF-κB signaling pathway may be a potential strategy for the treatment of RM-NPC.

Plasma EBV-DNA copy number is a validated tool for diagnosis, monitoring treatment response, and predicting patient survival in patients with NPC.[11,39–42] In contrast, the value of ctDNA requires much more study and validation before possible use in practice. Nevertheless, ctDNA not only reflects tumor burden but could also provide detailed information on genetic aberration, which in turn could be potentially useful in guiding therapeutic options. After all, both of the approaches generate quantitative data; thus, the selection of an appropriate threshold still needs to be clarified in future studies.

Limitations of the study

This trial has several limitations. First, the single-arm design of this study means there it no control group to make a direct comparison with other regimens. Second, the sample size is relatively small, particularly for analysis of predictive biomarkers. Randomized controlled trials of large sample size are needed to validate the effectiveness. Third, the ctDNA panel consisted of only 437 cancer-related genes; accordingly, some other mutations might have been missed.

Conclusion

This trial demonstrated the satisfactory antitumor efficacy and acceptable safety profile of the toripalimab and anlotinib regimen in patients with RM-NPC after failure of platinum-based therapy, and ctDNA could be used as a potential predictive biomarker after further validations.

RESOURCE AVAILABILITY

Lead contact

Further information and resource requests should be directly to and will be fulfilled by the lead contact, Qingqing Cai (caiqq@sysucc.org.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- De-identified individual patient-level data required to reanalyze the data are available from the lead contact upon request. Any additional information regarding individual participants that may result in a breach of patient confidentiality will not be provided. The raw sequence data reported in this paper have been deposited at Genome Sequence Archive[43] in National Genomics Data Center,[44] China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: HRA009204) and are publicly available as of the date of publication (accessible at https://ngdc.cncb.ac.cn/gsa).
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

This work was supported by grants from National Natural Science Foundation of China (81672686) and the China National Postdoctoral Program for Innovative Talents (BX20240445). The authors thank Shanghai Junshi Biosciences for donating toripalimab and Chia Tai Tianqing Pharmaceutical Group for donating anlotinib. Geneseeq Technology Inc. did the circulating tumor DNA sequencing. The authors thank Kehong Zhang, M.D., Ph.D., from Ivy Medical Editing (Shanghai, China) for revising the final manuscript. The graphical abstract was created with BioRender.com. Alluvial plots were created with Sangerbox.com.[45]

AUTHOR CONTRIBUTIONS

Conception and design, Q.C., Y.X., Y.Z., and Q.Z.; collection and assembly of data, Y.Z., Q.Z., and B.Z.; data analysis and interpretation, Q.C., Y.X., Y.Z., Q.Z., and B.Z.; writing – original draft, Y.Z. and Q.Z.; final approval of manuscript, all authors; funding acquisition, Q.C.

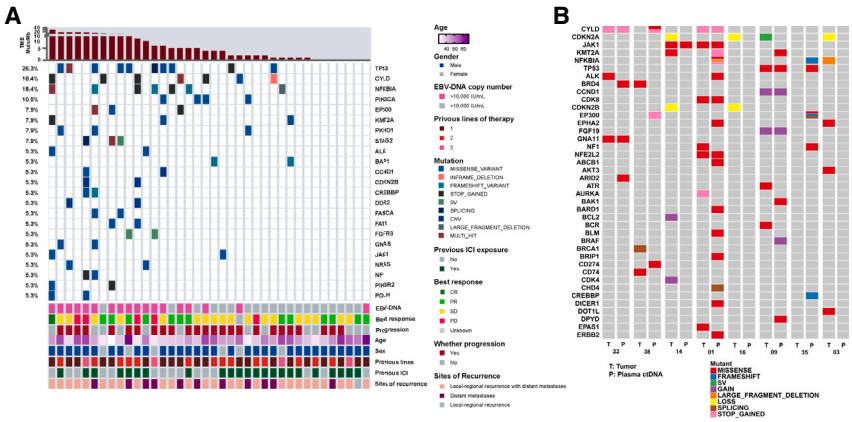
DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

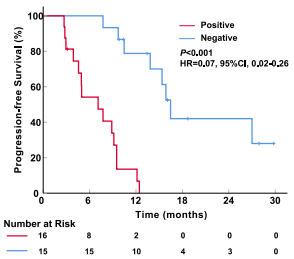
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE



C

Best Response	Negative (n=15)	Positive (n=16)
Objective response rate, n (%)	11 (73.3)	3 (18.8)
Best overall response, n (%)		
Complete response	1 (6.7)	0 (0.0)
Partial response	10 (66.7)	3 (18.8)
Stable disease	4 (26.7)	13 (81.3)
Progressive disease	0 (0.0)	0 (0.0)



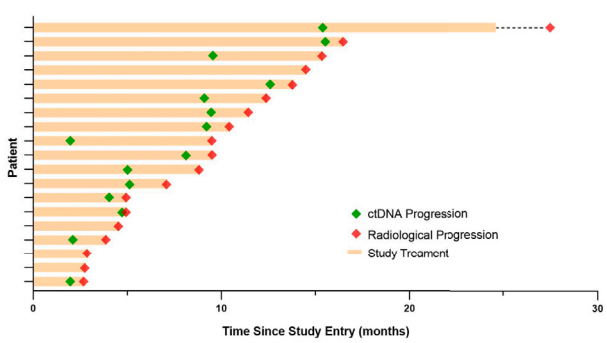


Figure 4. Exploratory analysis of ctDNA

(A) Heatmap plot of baseline ctDNA (n = 38; two patients without available peripheral blood samples were excluded).

(B) Heatmap plot of eight pairs of tumor tissue and ctDNA.

(C) Best response by bTMB at C2. Responses were evaluated according to RECIST version 1.1. Seven patients were further excluded in C2 analysis due to progressive disease after cycle 2 (n = 5), no available blood samples (n = 1), and no postbaseline assessment (n = 1).

(D) Kaplan-Meier estimate of PFS by bTMB at C2. The p value was determined by Log-rank test.

(E) Swimmer plot. bTMB, blood tumor mutation burden; ctDNA, circulating tumor DNA; HR, hazard ratio; ICI, immune checkpoint inhibitor; PFS, progression-free survival.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2024.101833.

Received: April 20, 2024
Revised: July 18, 2024
Accepted: November 4, 2024
Published: November 29, 2024

REFERENCES

1. Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., and Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA A Cancer J. Clin. *71*, 209–249. https://doi.org/10.3322/caac.21660.
2. Li, Y.Q., Tian, Y.M., Tan, S.H., Liu, M.Z., Kusumawidjaja, G., Ong, E.H.W., Zhao, C., Tan, T.W.K., Fong, K.W., Sommat, K., et al. (2018). Prognostic Model for Stratification of Radioresistant Nasopharynx Carcinoma to Curative Salvage Radiotherapy. J. Clin. Oncol. *36*, 891–899. https://doi.org/10.1200/jco.2017.75.5165.
3. Prawira, A., Oosting, S.F., Chen, T.W., Delos Santos, K.A., Saluja, R., Wang, L., Siu, L.L., Chan, K.K.W., and Hansen, A.R. (2017). Systemic therapies for recurrent or metastatic nasopharyngeal carcinoma: a systematic review. Br. J. Cancer *117*, 1743–1752. https://doi.org/10.1038/bjc.2017.357.
4. Chen, Y.P., Chan, A.T.C., Le, Q.T., Blanchard, P., Sun, Y., and Ma, J. (2019). Nasopharyngeal carcinoma. Lancet (London, England) *394*, 64–80. https://doi.org/10.1016/s0140-6736(19)30956-0.

5. Hong, S., Zhang, Y., Yu, G., Peng, P., Peng, J., Jia, J., Wu, X., Huang, Y., Yang, Y., Lin, Q., et al. (2021). Gemcitabine Plus Cisplatin Versus Fluorouracil Plus Cisplatin as First-Line Therapy for Recurrent or Metastatic Nasopharyngeal Carcinoma: Final Overall Survival Analysis of GEM20110714 Phase III Study. J. Clin. Oncol. *39*, 3273–3282. https://doi.org/10.1200/jco.21.00396.

6. Mai, H.Q., Chen, Q.Y., Chen, D., Hu, C., Yang, K., Wen, J., Li, J., Shi, Y.R., Jin, F., Xu, R., et al. (2021). Toripalimab or placebo plus chemotherapy as first-line treatment in advanced nasopharyngeal carcinoma: a multicenter randomized phase 3 trial. Nat. Med. *27*, 1536–1543. https://doi.org/10.1038/s41591-021-01444-0.

7. Yang, Y., Qu, S., Li, J., Hu, C., Xu, M., Li, W., Zhou, T., Shen, L., Wu, H., Lang, J., et al. (2021). Camrelizumab versus placebo in combination with gemcitabine and cisplatin as first-line treatment for recurrent or metastatic nasopharyngeal carcinoma (CAPTAIN-1st): a multicentre, randomised, double-blind, phase 3 trial. Lancet Oncol. *22*, 1162–1174. https://doi.org/10.1016/s1470-2045(21)00302-8.

8. Hsu, C., Lee, S.H., Ejadi, S., Even, C., Cohen, R.B., Le Tourneau, C., Mehnert, J.M., Algazi, A., van Brummelen, E.M.J., Saraf, S., et al. (2017). Safety and Antitumor Activity of Pembrolizumab in Patients With Programmed Death-Ligand 1-Positive Nasopharyngeal Carcinoma: Results of the KEYNOTE-028 Study. J. Clin. Oncol. *35*, 4050–4056. https://doi.org/10.1200/jco.2017.73.3675.

9. Ma, B.B.Y., Lim, W.T., Goh, B.C., Hui, E.P., Lo, K.W., Pettinger, A., Foster, N.R., Riess, J.W., Agulnik, M., Chang, A.Y.C., et al. (2018). Antitumor Activity of Nivolumab in Recurrent and Metastatic Nasopharyngeal Carcinoma: An International, Multicenter Study of the Mayo Clinic Phase 2 Consortium (NCI-9742). J. Clin. Oncol. *36*, 1412–1418. https://doi.org/10.1200/jco.2017.77.0388.

10. Fang, W., Yang, Y., Ma, Y., Hong, S., Lin, L., He, X., Xiong, J., Li, P., Zhao, H., Huang, Y., et al. (2018). Camrelizumab (SHR-1210) alone or in combination with gemcitabine plus cisplatin for nasopharyngeal carcinoma: results from two single-arm, phase 1 trials. Lancet Oncol. *19*, 1338–1350. https://doi.org/10.1016/s1470-2045(18)30495-9.

11. Wang, F.H., Wei, X.L., Feng, J., Li, Q., Xu, N., Hu, X.C., Liao, W., Jiang, Y., Lin, X.Y., Zhang, Q.Y., et al. (2021). Efficacy, Safety, and Correlative Biomarkers of Toripalimab in Previously Treated Recurrent or Metastatic Nasopharyngeal Carcinoma: A Phase II Clinical Trial (POLARIS-02). J. Clin. Oncol. *39*, 704–712. https://doi.org/10.1200/jco.20.02712.

12. Lim, W.T., Ng, Q.S., Ivy, P., Leong, S.S., Singh, O., Chowbay, B., Gao, F., Thng, C.H., Goh, B.C., Tan, D.S.W., et al. (2011). A Phase II study of pazopanib in Asian patients with recurrent/metastatic nasopharyngeal carcinoma. Clin. Cancer Res. *17*, 5481–5489. https://doi.org/10.1158/1078-0432.ccr-10-3409.

13. Hui, E.P., Ma, B.B.Y., Loong, H.H.F., Mo, F., Li, L., King, A.D., Wang, K., Ahuja, A.T., Chan, C.M.L., Hui, C.W.C., et al. (2018). Efficacy, Safety, and Pharmacokinetics of Axitinib in Nasopharyngeal Carcinoma: A Preclinical and Phase II Correlative Study. Clin. Cancer Res. *24*, 1030–1037. https://doi.org/10.1158/1078-0432.ccr-17-1667.

14. Zhang, Y., Luo, F., Ma, Y.X., Liu, Q.W., Yang, Y.P., Fang, W.F., Huang, Y., Zhou, T., Li, J., Pan, H.M., et al. (2022). A Phase Ib Study of Lucitanib (AL3810) in a Cohort of Patients with Recurrent and Metastatic Nasopharyngeal Carcinoma. Oncol. *27*, e453–e462. https://doi.org/10.1093/oncolo/oyab076.

15. Lin, B., Song, X., Yang, D., Bai, D., Yao, Y., and Lu, N. (2018). Anlotinib inhibits angiogenesis via suppressing the activation of VEGFR2, PDGFRβ and FGFR1. Gene *654*, 77–86. https://doi.org/10.1016/j.gene.2018.02.026.

16. Xie, C., Wan, X., Quan, H., Zheng, M., Fu, L., Li, Y., and Lou, L. (2018). Preclinical characterization of anlotinib, a highly potent and selective vascular endothelial growth factor receptor-2 inhibitor. Cancer Sci. *109*, 1207–1219. https://doi.org/10.1111/cas.13536.

17. Fang, Y., Su, N., Zou, Q., Cao, Y., Xia, Y., Tang, L., Tian, X., Liu, P., and Cai, Q. (2023). Anlotinib as a third-line or further treatment for recurrent or metastatic nasopharyngeal carcinoma: a single-arm, phase 2 clinical trial. BMC Med. *21*, 423. https://doi.org/10.1186/s12916-023-03140-x.

18. Ding, X., Zhang, W.J., You, R., Zou, X., Wang, Z.Q., Ouyang, Y.F., Peng, L., Liu, Y.P., Duan, C.Y., Yang, Q., et al. (2023). Camrelizumab Plus Apatinib in Patients With Recurrent or Metastatic Nasopharyngeal Carcinoma: An Open-Label, Single-Arm, Phase II Study. J. Clin. Oncol. *41*, 2571–2582. https://doi.org/10.1200/jco.22.01450.

19. Ding, X., Hua, Y.J., Zou, X., Chen, X.Z., Zhang, X.M., Xu, B., Ouyang, Y.F., Tu, Z.W., Li, H.F., Duan, C.Y., et al. (2023). Camrelizumab plus famitinib in patients with recurrent or metastatic nasopharyngeal carcinoma treated with PD-1 blockade: data from a multicohort phase 2 study. EClinicalMedicine *61*, 102043. https://doi.org/10.1016/j.eclinm.2023.102043.

20. Lu, N., Jiang, Y.F., Xia, W.X., Huang, Y., Xie, C.M., Xu, C., Ye, Y.F., Liu, G.Y., Bei, W.X., Ke, L.R., et al. (2023). Efficacy and safety of sintilimab plus bevacizumab in metastatic nasopharyngeal carcinoma after failure of platinum-based chemotherapy: an open-label phase 2 study. EClinicalMedicine *62*, 102136. https://doi.org/10.1016/j.eclinm.2023.102136.

21. Chan, A.T.C., Lee, V.H.F., Hong, R.L., Ahn, M.J., Chong, W.Q., Kim, S.B., Ho, G.F., Caguioa, P.B., Ngamphaiboon, N., Ho, C., et al. (2023). Pembrolizumab monotherapy versus chemotherapy in platinum-pretreated, recurrent or metastatic nasopharyngeal cancer (KEYNOTE-122): an open-label, randomized, phase III trial. Ann. Oncol. *34*, 251–261. https://doi.org/10.1016/j.annonc.2022.12.007.

22. Huang, Y., Yuan, J., Righi, E., Kamoun, W.S., Ancukiewicz, M., Nezivar, J., Santosuosso, M., Martin, J.D., Martin, M.R., Vianello, F., et al. (2012). Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. Proc. Natl. Acad. Sci. USA *109*, 17561–17566. https://doi.org/10.1073/pnas.1215397109.

23. Du Four, S., Maenhout, S.K., Niclou, S.P., Thielemans, K., Neyns, B., and Aerts, J.L. (2016). Combined VEGFR and CTLA-4 blockade increases the antigen-presenting function of intratumoral DCs and reduces the suppressive capacity of intratumoral MDSCs. Am. J. Cancer Res. *6*, 2514–2531.

24. Zhao, S., Ren, S., Jiang, T., Zhu, B., Li, X., Zhao, C., Jia, Y., Shi, J., Zhang, L., Liu, X., et al. (2019). Low-Dose Apatinib Optimizes Tumor Microenvironment and Potentiates Antitumor Effect of PD-1/PD-L1 Blockade in Lung Cancer. Cancer Immunol. Res. *7*, 630–643. https://doi.org/10.1158/2326-6066.cir-17-0640.

25. Chu, T., Zhong, R., Zhong, H., Zhang, B., Zhang, W., Shi, C., Qian, J., Zhang, Y., Chang, Q., Zhang, X., et al. (2021). Phase 1b Study of Sintilimab Plus Anlotinib as First-line Therapy in Patients With Advanced NSCLC. J. Thorac. Oncol. *16*, 643–652. https://doi.org/10.1016/j.jtho.2020.11.026.

26. Wei, W., Ban, X., Yang, F., Li, J., Cheng, X., Zhang, R., Huang, X., Huang, Y., Li, Q., Qiu, Y., et al. (2022). Phase II trial of efficacy, safety and biomarker analysis of sintilimab plus anlotinib for patients with recurrent or advanced endometrial cancer. J. Immunother. Cancer *10*, e004338. https://doi.org/10.1136/jitc-2021-004338.

27. Xu, Q., Wang, J., Sun, Y., Lin, Y., Liu, J., Zhuo, Y., Huang, Z., Huang, S., Chen, Y., Chen, L., et al. (2022). Efficacy and Safety of Sintilimab Plus Anlotinib for PD-L1-Positive Recurrent or Metastatic Cervical Cancer: A Multicenter, Single-Arm, Prospective Phase II Trial. J. Clin. Oncol. *40*, 1795–1805. https://doi.org/10.1200/jco.21.02091.

28. Qin, B.D., Jiao, X.D., Wang, Z., Liu, K., Wu, Y., Ling, Y., Chen, S.Q., Zhong, X., Duan, X.P., Qin, W.X., et al. (2023). Pan-cancer efficacy and safety of anlotinib plus PD-1 inhibitor in refractory solid tumor: A single-arm, open-label, phase II trial. Int. J. Cancer *153*, 815–825. https://doi.org/10.1002/ijc.34546.

29. Wang, Z., Duan, J., Cai, S., Han, M., Dong, H., Zhao, J., Zhu, B., Wang, S., Zhuo, M., Sun, J., et al. (2019). Assessment of Blood Tumor Mutational Burden as a Potential Biomarker for Immunotherapy in Patients With Non-Small Cell Lung Cancer With Use of a Next-Generation Sequencing Cancer Gene Panel. JAMA Oncol. *5*, 696–702. https://doi.org/10.1001/jamaoncol.2018.7098.

30. Zhang, Q., Luo, J., Wu, S., Si, H., Gao, C., Xu, W., Abdullah, S.E., Higgs, B.W., Dennis, P.A., van der Heijden, M.S., et al. (2020). Prognostic and Predictive Impact of Circulating Tumor DNA in Patients with Advanced Cancers Treated with Immune Checkpoint Blockade. Cancer Discov. *10*, 1842–1853. https://doi.org/10.1158/2159-8290.cd-20-0047.

31. Pan, Y., Zhang, J.T., Gao, X., Chen, Z.Y., Yan, B., Tan, P.X., Yang, X.R., Gao, W., Gong, Y., Tian, Z., et al. (2023). Dynamic circulating tumor DNA during chemoradiotherapy predicts clinical outcomes for locally advanced non-small cell lung cancer patients. Cancer Cell *41*, 1763–1773.e4. https://doi.org/10.1016/j.ccell.2023.09.007.

32. You, R., Zou, X., Ding, X., Zhang, W.J., Zhang, M.X., Wang, X., Xu, H.S., Liu, Y.L., Ouyang, Y.F., Duan, C.Y., et al. (2022). Gemcitabine combined with apatinib and toripalimab in recurrent or metastatic nasopharyngeal carcinoma. Méd. *3*, 664–681.e6. https://doi.org/10.1016/j.medj.2022.07.009.

33. Han, Y., Wang, J., Sun, T., Ouyang, Q., Li, J., Yuan, J., and Xu, B. (2023). Predictive biomarkers of response and survival following immunotherapy with a PD-L1 inhibitor benmelstobart (TQB2450) and antiangiogenic therapy with a VEGFR inhibitor anlotinib for pretreated advanced triple negative breast cancer. Signal Transduct. Targeted Ther. *8*, 429. https://doi.org/10.1038/s41392-023-01672-5.

34. Zheng, H., Dai, W., Cheung, A.K.L., Ko, J.M.Y., Kan, R., Wong, B.W.Y., Leong, M.M.L., Deng, M., Kwok, T.C.T., Chan, J.Y.W., et al. (2016). Whole-exome sequencing identifies multiple loss-of-function mutations of NF-κB pathway regulators in nasopharyngeal carcinoma. Proc. Natl. Acad. Sci. USA *113*, 11283–11288. https://doi.org/10.1073/pnas.1607606113.

35. Li, Y.Y., Chung, G.T.Y., Lui, V.W.Y., To, K.F., Ma, B.B.Y., Chow, C., Woo, J.K.S., Yip, K.Y., Seo, J., Hui, E.P., et al. (2017). Exome and genome sequencing of nasopharynx cancer identifies NF-κB pathway activating mutations. Nat. Commun. *8*, 14121. https://doi.org/10.1038/ncomms14121.

36. You, R., Liu, Y.P., Lin, D.C., Li, Q., Yu, T., Zou, X., Lin, M., Zhang, X.L., He, G.P., Yang, Q., et al. (2019). Clonal Mutations Activate the NF-κB Pathway to Promote Recurrence of Nasopharyngeal Carcinoma. Cancer Res. *79*, 5930–5943. https://doi.org/10.1158/0008-5472.can-18-3845.

37. Li, Y., Shi, F., Hu, J., Xie, L., Zhao, L., Tang, M., Luo, X., Ye, M., Zheng, H., Zhou, M., et al. (2021). Stabilization of p18 by deubiquitylase CYLD is pivotal for cell cycle progression and viral replication. npj Precis. Oncol. *5*, 14. https://doi.org/10.1038/s41698-021-00153-8.

38. Wang, L., Lin, Y., Zhou, X., Chen, Y., Li, X., Luo, W., Zhou, Y., and Cai, L. (2022). CYLD deficiency enhances metabolic reprogramming and tumor progression in nasopharyngeal carcinoma via PFKFB3. Cancer Lett. *532*, 215586. https://doi.org/10.1016/j.canlet.2022.215586.

39. Tang, L.Q., Chen, Q.Y., Fan, W., Liu, H., Zhang, L., Guo, L., Luo, D.H., Huang, P.Y., Zhang, X., Lin, X.P., et al. (2013). Prospective study of tailoring whole-body dual-modality [18F]fluorodeoxyglucose positron emission tomography/computed tomography with plasma Epstein-Barr virus DNA for detecting distant metastasis in endemic nasopharyngeal carcinoma at initial staging. J. Clin. Oncol. *31*, 2861–2869. https://doi.org/10.1200/jco.2012.46.0816.

40. Hui, E.P., Li, W.F., Ma, B.B., Lam, W.K.J., Chan, K.C.A., Mo, F., Ai, Q.Y.H., King, A.D., Wong, C.H., Guo, R., et al. (2020). Integrating postradiotherapy plasma Epstein-Barr virus DNA and TNM stage for risk stratification of nasopharyngeal carcinoma to adjuvant therapy. Ann. Oncol. *31*, 769–779. https://doi.org/10.1016/j.annonc.2020.03.289.

41. Wang, W.Y., Twu, C.W., Chen, H.H., Jan, J.S., Jiang, R.S., Chao, J.Y.C., Liang, K.L., Chen, K.W., Wu, C.T., and Lin, J.C. (2010). Plasma EBV DNA clearance rate as a novel prognostic marker for metastatic/recurrent nasopharyngeal carcinoma. Clin. Cancer Res. *16*, 1016–1024. https://doi.org/10.1158/1078-0432.ccr-09-2796.

42. Chan, A.T.C., Hui, E.P., Ngan, R.K.C., Tung, S.Y., Cheng, A.C.K., Ng, W.T., Lee, V.H.F., Ma, B.B.Y., Cheng, H.C., Wong, F.C.S., et al. (2018). Analysis of Plasma Epstein-Barr Virus DNA in Nasopharyngeal Cancer After Chemoradiation to Identify High-Risk Patients for Adjuvant Chemotherapy: A Randomized Controlled Trial. J. Clin. Oncol. *36*, Jco2018777847. https://doi.org/10.1200/jco.2018.77.7847.

43. Chen, T., Chen, X., Zhang, S., Zhu, J., Tang, B., Wang, A., Dong, L., Zhang, Z., Yu, C., Sun, Y., et al. (2021). The Genome Sequence Archive Family: Toward Explosive Data Growth and Diverse Data Types. Dev. Reprod. Biol. *19*, 578–583. https://doi.org/10.1016/j.gpb.2021.08.001.

44. CNCB-NGDC Members and Partners (2024). Database Resources of the National Genomics Data Center, China National Center for Bioinformation in 2024. Nucleic Acids Res. *52*, D18-d32. https://doi.org/10.1093/nar/gkad1078.

45. Shen, W., Song, Z., Zhong, X., Huang, M., Shen, D., Gao, P., Qian, X., Wang, M., He, X., Wang, T., et al. (2022). Sangerbox: A comprehensive, interaction-friendly clinical bioinformatics analysis platform. iMeta *1*, e36. https://doi.org/10.1002/imt2.36.

46. Niu, B., Ye, K., Zhang, Q., Lu, C., Xie, M., McLellan, M.D., Wendl, M.C., and Ding, L. (2014). MSIsensor: microsatellite instability detection using paired tumor-normal sequence data. Bioinformatics *30*, 1015–1016. https://doi.org/10.1093/bioinformatics/btt755.

47. Faust, G.G., and Hall, I.M. (2014). SAMBLASTER: fast duplicate marking and structural variant read extraction. Bioinformatics *30*, 2503–2505. https://doi.org/10.1093/bioinformatics/btu314.

48. Wang, K., Li, M., and Hakonarson, H. (2010). ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. *38*, e164. https://doi.org/10.1093/nar/gkq603.

49. Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics *25*, 1754–1760. https://doi.org/10.1093/bioinformatics/btp324.

50. Koboldt, D.C., Zhang, Q., Larson, D.E., Shen, D., McLellan, M.D., Lin, L., Miller, C.A., Mardis, E.R., Ding, L., and Wilson, R.K. (2012). VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. *22*, 568–576. https://doi.org/10.1101/gr.129684.111.

51. Eisenhauer, E.A., Therasse, P., Bogaerts, J., Schwartz, L.H., Sargent, D., Ford, R., Dancey, J., Arbuck, S., Gwyther, S., Mooney, M., et al. (2009). New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur. J. Cancer *45*, 228–247. https://doi.org/10.1016/j.ejca.2008.10.026.

52. Chalmers, Z.R., Connelly, C.F., Fabrizio, D., Gay, L., Ali, S.M., Ennis, R., Schrock, A., Campbell, B., Shlien, A., Chmielecki, J., et al. (2017). Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med. *9*, 34. https://doi.org/10.1186/s13073-017-0424-2.

53. Wang, F., Wei, X.L., Feng, J., Li, Q., Xu, N., Hu, X., Liao, W., Jiang, Y., Lin, X., Zhang, Q., et al. (2020). Clinical response and biomarker analysis of POLARIS-02 a phase II study of toripalimab, a humanized IgG4 mAb against programmed death-1 (PD-1) in patients with metastatic nasopharyngeal carcinoma. J. Clin. Oncol. *38*, 6542. https://doi.org/10.1200/JCO.2020.38.15_suppl.6542.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human plasma	this study	N/A
Human tumor tissue	this study	N/A
Critical commercial assays		
DNeasy Blood and Tissue Kit	Qiagen	#69506
QIAamp Circulating Nucleic Acid Kit	Qiagen	#55114
dsDNA HS Assay Kit	Life Technologies	#Q32854
KAPA Hyper Prep Kit	KAPA Biosystems	#KK8504/KK8529
Deposited data		
The Genome Sequence Archive (GSA)	this study	GSA: HRA009204
Software and algorithms		
MSIsensor	Niu et al.[46]	https://github.com/ding-lab/msisensor
SAMBLASTER (v0.1.21)	Faust et al.[47]	https://github.com/GregoryFaust/samblaster
ANNOVAR (v 2015Mar22)	Wang et al.[48]	https://annovar.openbioinformatics.org/en/latest/
BWA (v0.7.12)	Li et al.[49]	http://bio-bwa.sourceforge.net/
VarScan2	Koboldt et al.[50]	http://dkoboldt.github.io/varscan/
Picard (V.1.119)	Broad Institute	https://github.com/broadinstitute/picard
R software (v4.0.2)	R project	https://www.r-project.org/
SPSS software 25.0	IBM	https://www.ibm.com/spss
GraphPad Prism 8.0.2	GraphPad Prism	https://www.graphpad.com/scientific-software/prism/
BioRender	BioRender website	https://www.biorender.com/
Sangerbox	Sangerbox website	https://www.sangerbox.com/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study design and participants

This is an investigator-initiated, multi-center, single-arm, phase 2 trial to evaluate the clinical activity and safety of toripalimab plus anlotinib in patients with RM-NPC. The trial was approved by the ethics committee of Sun Yat-sen University Cancer Center (B2021-055), Sun Yat-sen Memorial Hospital (SYSKY-2022-506-01), and The First Affiliated Hospital of Guangdong Pharmaceutical University (2022-68). The trial was conducted in compliance with the Declaration of Helsinki. All participants provided written informed consent prior to enrollment.

Adult patients (18–70 years of age) with histologically confirmed non-keratinizing, undifferentiated NPC who failed at least one line of platinum-based systemic chemotherapy, or progressed within 6 months after concurrent chemoradiotherapy were eligible. Other key eligibility criteria included at least one measurable lesion according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1,[51] Eastern Cooperative Oncology Group performance status of 0–2, and adequate organ function. Key exclusion criteria included prior treatment with bevacizumab or other TKIs targeting VEGF(R), tumor invasion to major vessels, any grade $\geq$3 hemorrhages within 4 weeks before enrollment, and symptomatic central nervous system metastases. The full eligibility criteria are listed in trial protocol (Supplemental materials).

METHOD DETAILS

Study treatment and assessments

Patients received toripalimab 240 mg intravenously once every 3 weeks and anlotinib 12 mg orally once daily (2 weeks on and 1 week off), until disease progression, intolerable toxicities, death, or at patients' request. Dose modifications of toripalimab were not

allowed. Dose modifications of anlotinib were allowed for anlotinib-related toxicities that were not relieved by supporting and symptomatic treatment, with a first dose reduction to 10 mg and a second reduction to 8 mg.

Tumor responses were assessed every two cycles according to RECIST v1.1. Adverse events (AE) were graded every cycle according to the National Cancer Institute Common Terminology Criteria v5.0.

Endpoints

The primary endpoint was ORR, defined as the proportion of patients achieving CR or PR according to RECIST v1.1. The secondary endpoints included DCR, defined as the proportion of patients achieving CR, PR, or SD; PFS, defined as the time from study entry to disease progression or death; OS, defined as the time from study entry to death from any cause; DOR, defined as the time from initially confirmed response to disease progression or death; and safety.

Efficacy was evaluated in both the ITT population and efficacy analysis population (had at least one postbaseline tumor assessment). Safety was evaluated in all the patients receiving at least one cycle of study regimen.

Circulating tumor DNA analysis

(1) Sample collection and DNA extraction

Peripheral blood samples were collected before treatment initiation/baseline (C0), at the end of cycle 1 and 2 (C1 & C2), and thereafter every 2 cycles until disease progression (Figure S4). Peripheral blood samples (8mL) were collected in ethylenediaminetetraacetic acid tubes before treatment initiation (C0), at the end of cycle 1/2 (C1/C2), and thereafter every 2 cycles until radiological disease progression. Formalin-fixed paraffin-embedded (FFPE) tumor samples were collected from 8 patients (6 at the initial diagnosis and 2 at the time of trial enrollment) to examine the concordance of mutation profiles between tumor tissue and ctDNA.

Peripheral blood samples were processed within 2 h to separate peripheral blood cells and plasma by centrifugation (1400g, 4°C for 15 min), and shipped to the central testing laboratory (Nanjing Geneseeq Technology Inc.) within 48 h. Cell free DNA was extracted using the QIAamp Circulating Nucleic Acid Kit (QIAGEN). Germline genomic DNA was extracted from peripheral blood lymphocytes using the DNeasy Blood & Tissue kit (QIAGEN) as control for germline mutations. DNA concentration was quantified by dsDNA HS Assay Kit (Life Technologies, Eugene, OR, USA) according to the manufacturer's recommendations.

(2) Library Preparation and sequencing

Sequencing libraries were prepared using the KAPA Hyper Prep kit (KAPA Biosystems, Cape Town, South Africa) with an optimized manufacturer's protocol. In brief, 50ng - 1 μg of genomic DNA was sheared into 350 bp fragments using the Bioruptor Pico ，and then underwent end-repairing, A-tailing and ligation with indexed adapters sequentially, followed by size selection using Agencourt AMPure XP beads. Finally, libraries were amplified by PCR and purified for target enrichment.

Different libraries with unique indices were pooled together in desirable ratios for up to 2 μg of total library input. Human cot-1 DNA (Life Technologies, Carlsbad, CA, USA) and xGen Universal blocking oligos (Integrated DNA Technologies, Coralville, IA, USA) targeting 437 cancer related genes were used for hybridization enrichment. The capture reaction was performed with the NimbleGen SeqCap EZ Hybridization and Wash Kit (Roche, Madison, WI, USA) and Dynabeads M-270 (Life Technologies, Vilnius, Lithuania) with optimized manufacturers' protocols. Captured libraries were on-beads amplified with Illumina p5 and p7 primers in KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Cape Town, South Africa). The post-capture amplified library was purified by Agencourt AMPure XP beads and quantified by qPCR using the KAPA Library Quantification kit (KAPA Biosystems, Cape Town, South Africa). Library fragment size was determined by the Agilent Technologies 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Enriched libraries were sequenced on Hiseq 4000 NGS platforms (Illumina) to targeted mean coverage depths of at least 300x for whole blood control samples, 1000x for FFPE samples, and 9000x for cfDNAs.

(3) Sequence Data Processing

Trimmomatic was used for FASTQ file quality control (QC). Reads from each sample were mapped to the reference sequence hg19 (Human Genome version 19) using Burrows-Wheeler Aligner (BWA-mem, v0.7.12). VarScan2 was employed for detection of somatic mutations. Annotation was performed using ANNOVAR on hg19 reference genome and 2014 versions of standard databases and functional prediction programs. Somatic Single Nucleotide Variant (SNV) calling was performed using Mutect and insetion/deletions (INDELs) were called running Scalpel (scalpel-discovery in–somatic mode). Genomic fusions were identified by FACTERA with default parameters. Copy number variations (CNVs) were detected using ADTEx (http://adtex.sourceforge.net) with default parameters. SNVs and INDELs called were further filtered using the following criteria: (a) minimum $\geq$2 variant supporting reads and $\geq$0.03% variant allele frequency (VAF) supporting the variant, (b) filtered if present in >1% population frequency in the 1000g or ExAC database, (c) filtered through an internally collected list of recurrent sequencing errors ($\geq$3 variant reads and $\leq$20% VAF in at least 30 out of ~2,000 normal samples) on the same sequencing platform. Final list of mutations was annotated using vcf2maf (call VEP for annotation). Tumor mutation burden (TMB) was defined as the total number of missense mutations, and was counted by summing all base substitutions and INDELs in the coding region of targeted genes, including synonymous alterations to reduce sampling noise and excluding known driver mutations as they are over-represented in the panel.[52]

(4) Clinical relevance analysis

Longitudinally monitoring of bTMB was performed to evaluate the value of ctDNA as a biomarker for prediction of treatment response, survival outcomes, and disease progression. Positive ctDNA was defined as bTMB >0 muts/Mb. A "significant increase" of ctDNA was defined as a fold increase $\geq$100% or two successive increases of bTMB during treatment.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample size estimation

Sample size requirement was estimated according to a single-stage phase 2 design (power of 80% and a type I error of 5%). An ORR less than 20.5% was considered ineffective, which was referenced from the phase 2 trial of toripalimab monotherapy for RM-NPC (POLARIS-02),[53] and the target ORR was set to 40%. The estimated sample size was 35, and considering 10% dropout, we planned to enroll 39 patients.

Statistical analysis

Descriptive statistics were used to present baseline characteristics, safety, and other clinical data. The ORR, DCR, and their 95% CI were calculated using Clopper–Pearson method, and compared by Fisher's exact test. The PFS, OS, and DOR were estimated using Kaplan-Meier method and compared by log rank test. Hazard ratio (HR) was calculated using univariate Cox regression analysis. Statistical analyses were performed using SPSS software (version 25.0), R software (version 4.0.2), and GraphPad Prism software (version 8.0.2). Differences were considered statistically significant at $p < 0.05$.

ADDITIONAL RESOURCES

This study has been registered on "https://clinicaltrials.gov/," ID: NCT04996758.