eLife

Reviewed Preprint
v2 • September 30, 2025
Revised by authors

Reviewed Preprint
v1 • November 26, 2024

Cancer Biology

Low-dose chemotherapy combined with delayed immunotherapy in the neoadjuvant treatment of non-small cell lung cancer and dynamic monitoring of the drug response in peripheral blood

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eLife Assessment

Liang et al. have conducted a small pilot study investigating the feasibility and tolerability of a regimen of neoadjuvant chemo-immunotherapy for non-small cell lung cancer, with lower cumulative dose of chemotherapy and with the immunotherapy delivered on D8 of each cycle. The clinical data are interesting and novel, and overall the findings of the study are **valuable**. However, the translational data and analyses are **incomplete** and do not support key claims in the title.

https://doi.org/10.7554/eLife.99720.2.sa3

Abstract

Background

Despite chemo-immunotherapy has been applied to the neoadjuvant treatment of non-small cell lung cancer (NSCLC), the impacts of dosage and the order of medication on treatment efficacy and safety remain largely unexplored. We originally designed an exploratory study to investigate the efficacy and safety of reduced-dose chemotherapy combined with delayed immunotherapy as well as the dynamic changes of circulating tumor DNA (ctDNA) and T cell receptor (TCR) during the therapy.

Methods

Patients with clinical stage IIA to IIIA resectable NSCLC were treated with 2 cycles of reduced-dose platinum-based chemotherapy on day 1 combined with immunotherapy on day 5. The same postoperative modified adjuvant therapy regimen was administered for 2 cycles. Plasma samples at different time-points were collected and performed with T cell receptor (TCR) and circulating tumor DNA (ctDNA) sequencing.

Results

38 patients received modified chemo-immunotherapy. The proportion of patients exhibiting complete response and partial response was 5.3% and 68.4%, respectively. The confirmed objective response rate was 73.7%. Radiological downstaging was achieved in 39.5%. Major pathologic response and complete pathologic response were observed in 47.4% and 31.6% of patients, respectively. Only one patient experienced grade 3 adverse event. Further analyses revealed that this modified chemo-immunotherapy led to the expansion of predominant TCR clones and reduction of tumor burden after the first cycle of chemotherapy.

Conclusion

The promising clinical efficacy and low side effects of modified neoadjuvant chemo-immunotherapy position it as a prospective and innovative strategy for NSCLC.

Trial registration

Registration Number: ChiCTR2000033092

Background

Approximately 20 to 25% of patients diagnosed with NSCLC are eligible for surgical resection (1), yet 30% to 55% of those who undergo curative surgery will encounter recurrence and ultimately die (2, 3). Despite the improvement in treatment of NSCLC patients, the 5-year survival rates remain relatively low, hovering around 36% (4). Over the past decade, neoadjuvant platinum-based chemotherapy, followed by surgery, has become the standard of care. Nevertheless, only 5 to 6 % improvements have been noted in 5-year recurrence-free survival and overall survival rates compared to surgery alone (5).

Currently, combination of chemotherapy with immunotherapy has demonstrated superior clinical efficacy and prolonged event-free survival for patients with resectable NSCLC. Trials such as CheckMate 816 (6), SAKK 16/14 (7), and NADIM (8), have showcased the enhanced efficacy and manageable safety of combination therapy compared to monotherapy. However, for most previous clinical trials, cytotoxic drugs with standard doses and anti-PD1 antibody were administrated on the same day (9), which may result in unsatisfactory eradication rates and relatively high incidence of severe treatment-related adverse events (TRAEs). To improve the cure rate and reduce TRAEs, the use of low-dose chemotherapy may represent a feasible alternative,

given it favorable tolerability profile without compromising efficacy (10, 11). Although various regimens of low-dose chemotherapy have been utilized in cancer therapy (12, 13), the effects of dosage and administration sequence on the efficacy and safety of NSCLC treatment remain controversial. According to our previous research findings, the administration of immunotherapy 3-5 days after chemotherapy significantly reduces lymphocyte damage (14). Therefore, we hypothesize that by coordinating the dosage of chemotherapy and the timing of immunotherapy administration, we can enhance the synergistic effects between the two interventions. Ultimately, this could improve clinical efficacy while reducing toxic side effects.

To monitor the therapeutic efficacy during the process of chemo-immunotherapy, there is an urgent requirement for reliable indicators. The T cell receptor (TCR) repertoire and the presence of circulating tumor DNA (ctDNA) in blood have been proven to be efficient predictors of therapeutic responses in cancer treatment (15, 16). Chemotherapeutic agents have the capability to induce immunogenic death of tumor cells, reduce tumor burden, and promote the infiltration of CD8 T lymphocyte (17, 18). The recognition of tumor antigens by CD8 T lymphocytes relies primarily on the presence of a diverse and polymorphic TCR within an individual (19). Hence, it is imperative to explore the dynamic changes of TCR diversity and tumor burden in ctDNA throughout the course of therapy.

In this study, we report the results of our pilot clinical trial assessing the efficacy and safety of modified neoadjuvant chemo-immunotherapy in patients with resectable NSCLC. These results indicate that the combination of low-dose chemotherapy followed by delayed immunotherapy could potentially induce tumor cell damage, promote antigen exposure, and support T cell expansion, thereby enhancing therapeutic efficacy and reducing toxic side effects. Monitoring the dynamic changes of TCR repertoire and ctDNA does not only offer insights into the mechanism underlying our therapeutic approach to some extent but also furnishes valuable data for monitoring treatment efficacy.

Materials and methods

Study design and participants

38 Patients with resectable stage IIA to IIIA NSCLC, as per the staging criteria outlined by the American Joint Committee on Cancer, 8th edition, and possessing adequate organ function, along with an Eastern Cooperative Oncology Group performance-status rating of 0 to 1, were included in this study. All patients had not received any prior cancer treatment, exhibited measurable disease according to the Response Evaluation Criteria in Solid Tumors (version 1.1) and had confirmed histological or cytological evidence of the disease. Exclusion criteria included the presence of identified *EGFR* mutations or *ALK* translocations; a diagnosis of malignancies beyond NSCLC within 5 years; previous exposure to specific therapies such as anti-PD-1, anti-PD-L1, or anti-PD-L2 agents or medications targeting T cell receptor modulation (e.g., CTLA-4, LAG-3, or TIM-3); a history of allogeneic organ or hematopoietic stem cell transplantation (except corneal transplantation); ongoing repercussions from prior interventions; pregnancy or breastfeeding; and HIV positivity. Moreover, the availability of pre- and post-adjuvant treatment tumor tissues and serial blood samples facilitated comprehensive analyses including whole transcriptome sequencing, TCR sequencing, and ctDNA sequencing.

Trial design and treatment

This study was a single-center, single-arm, pilot clinical trial (Registration Number: ChiCTR2000033092). Eligible patients received 2 cycles of neoadjuvant therapy of low-dose platinum-based chemotherapy: albumin-bound paclitaxel 180-220 mg/m^2 plus cisplatin 60 mg/m^2 for lung squamous cell carcinoma or pemetrexed disodium 500 mg/m^2 plus cisplatin 60 mg/m^2 for lung adenocarcinoma respectively on day 1 plus sintilimab 200 mg on day 5 of each cycle, followed

by radiographic evaluation before undergoing radical surgery. Surgery was conducted within 6 weeks after the completion of neoadjuvant treatment. Then patients would receive 2 cycles of adjuvant therapy with the same regimen.

Study outcomes

The primary endpoints include objective response rate (ORR: the proportion of patients with complete response (CR) or partial response (PR) according to response evaluation criteria in solid tumors) and major pathologic response (MPR) (less than 10% residual viable tumor cells in the primary tumor and sampled lymph nodes) according to the pathologist review. Secondary endpoint included pathologic complete response (pCR) (0% residual viable tumor cells in the primary tumor and sampled lymph nodes), disease free survival (DFS), treatment-related adverse events (TRAEs), occurrence of surgical complications, and intraoperative and perioperative complications.

Plasma collection, cell-free DNA extraction, and sequencing

Blood samples were collected into cell-free DNA blood streck tubes. Isolated plasma and paired white blood cell (WBC) control were stored at -80 until extraction. Cell-free DNA (cfDNA) and WBC-derived DNA were extracted from 4 mL plasma and WBC using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and Mag-Bind® Blood & Tissue DNA HDQ 96 kit (OMEGA) according to the manufacturer's instructions, respectively. DNA extracts were sheared to 100 bp fragments using an S220 focused ultrasonicator (Covaris, Woodbrun, MA, USA). The adaptors (NadPrep®) were attached to the terminal of fragments and followed by PCR (Bio-Rad T100, USA) replication. The libraries were constructed using NadPrep Plasma Cell-Free DNA Dual-Ended Molecular Tag Library Construction Kit (for MGI) (Nanodigmbio). The target customized probes YuceOne Plus (ctDNA) synthesized by IDT were used for hybridization enrichment. Target enrichment was performed with NadPrep Hybrid Capture Reagents (Nanodigmbio). The enriched DNA fragments were sequenced with the MGISEQ platform. The mean depth of coverage was 3445× for cfDNA and 1419× for matched peripheral blood samples.

Circulating tumor DNA (ctDNA) analysis

SOAPnuke was implemented to cut the adapter and remove low-quality raw reads (20). Genecore was performed for cluster reads by unique molecule index (UMI) and remove the replicated reads derived from PCR (21). Clean reads were aligned against the human reference genome (hg19) with BWA (v0.7.12) (22) and duplicated reads were removed by samtools (v0.1.19) (23). Subsequently, generated BAM files were used for downstream analysis. We compared the ctDNA samples and matched control blood sequencing data to identify the somatic mutations, including single nucleotide variants (SNVs) and small insertions and deletions (Indels) using mutation caller Vardict (v1.7.0) with default parameters (24). Next, the caller was run with dbSNP (version 147), 1000G (phase3_release_v5), CLINVAR (version 151 ClinVar (*nih.gov*)), and COSMIC (version 81) data for known polymorphic sites. Substitutions and indels would be filtered out according to the following criteria: 1) low variant allelic fractions (VAF < 0.0005), 2) removed any variants with greater than 1 supporting read in the matched germline sample, 3) variants present in the above genome database. In addition, the filtered mutations were annotated by snpEff (v4.3) (25) with NCBIrefseq (*https://www.ncbi.nlm.nih.gov/refseq/*). The maximum VAF is considered as the tumor burden during the therapy.

Whole transcriptome sequencing

The total RNA of tumor samples was isolated using the RNeasy FFPE Kit (Qiagen, GER) according to the manufacturer's instructions. The concentrations of RNA were quantified using Qubit™ RNA HS Assay Kit (ThermoFisher Scientific, USA). RNA purity and integrity were checked using the Take3 (BioTek, USA) and the RNA Cartridge kit of the Qseq100 Bio-Fragment Analyzer (Bioptic, CHN),

respectively. Then, RNA sequencing (RNA-seq) libraries were constructed using the rRNA depletion module (H/M/R) and NadPrep DNA Library Preparation Module for MGI (MGI, CHN). The libraries were sequenced on the MGISEQ-T7 platform with 100 bp paired-end reads.

RNA-Seq raw data quality control, gene expression analysis, and immune cell abundance estimation

Raw sequencing data (raw reads) from MGISEQ-T7 was processed to filter out low-quality reads. Clean reads from each sample were aligned against the hg19 reference by STAR (v2.7.8) (26). The read counts and transcripts per million (TPM) values were calculated based on aligned RNA sequencing reads to reference transcripts downloaded from gencode (v27lift37) database, as implemented in rsem (v1.3.0) (27). Based on the gene expression matrix, R packages xCell (28) and web tool ImmunCellAI (29) were used to estimate the infiltration abundance of immune cells for each sample.

T cell receptor sequencing and analysis

For T cell receptor (TCR) sequencing, total RNA was isolated from peripheral blood mononuclear cells (PBMCs) by RNeasy Plus Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Qubit™ RNA HS Assay Kit (ThermoFisher Scientific, USA) was applied to determine the final concentration. Then TCR β-chain of CDR3 was amplified by Multi-IR Library Prep Kit (iGeneTech, CHN). Sequencing procedures were utilized by the MGISEQ-T7 platform with 100-bp paired-end reads. Fastq reads were trimmed based on their low-quality 3' ends to base. Trimmed pair-end reads were integrated according to overlapping alignment with the modified Needleman-Wunsch algorithm. MiXCR (v2.1.10) (30) was performed to identify the CDR3 sequences of V-D-J gene segments with reference sequences from the IMGT (31). VDJtools (v1.2.1) (32) was utilized to assess the immune repertoire sequencing. A frequency-based correction was performed on samples. The diversity of the TCR repertoire was calculated with the Shannon-Wiener index formula represents the frequency of clonotype i for the sample with n unique clonotypes. The evenness of the TCR repertoire was calculated with the formula , where H was Shannon Weiner index and S was the total number of species in a sample, across all samples in the dataset. Clonality is defined as a normalized Shannon index over n unique clones: clonality = 1 − H/ln(n). The D50 index was also performed to evaluate the diversity according to the top 50% of the total TCR clones in the samples. According to clonotype abundance, TCR clonotypes were defined as large clones when their frequency > 0.001. Based on the frequency ranking, the top 20 most frequent TCR clones and large clones are defined as the predominant TCR clones.

Statistical analysis

All statistical analyses were implemented by R 4.0 software or SPSS 27.0. Medians and inter quartile range (IQRs) were provided for distributions of observation duration and time intervals. Wilcoxon's Rank-Sum test was used to compare across independent groups. Wilcoxon's Signed-Rank test was used for the pairwise comparison samples. All reported *P* values are two-sided, and *P* values less than 0.05 were considered statistically significant.

Results

Overview of the patient cohort

From May 2020 to November 2023, 50 patients diagnosed with NSCLC at stage IIA-IIIA were screened for eligibility, of which 38 received two cycles of modified neoadjuvant chemo-immunotherapy (**Fig. 1A**). Each cycle featured two phases, corresponding to chemotherapy and immunotherapy (**Fig. 1B**). Following modified neoadjuvant chemo-immunotherapy, 31 patients underwent R0 resection (**Figure 1A** and **Table S1**). The postoperative tissues were subjected

to pathological evaluation. Samples from a representative patient (P2) who achieved pCR after neoadjuvant treatment exhibited morphological alterations, characterized by extracellular mucin pools and fibrosis (**Fig. 1C**). Computed tomography scans demonstrated the obliteration of lung lesions following neoadjuvant therapy (**Fig. 1D**). 74.2% (27/31) participants received the modified adjuvant chemo-immunotherapy (from time-point c3d0 to c4d21) (**Fig. 1A and B**; **Table S1**). Detailed treatment regimens are provided in **Table S2**.

Most patients were men (78.7%) and had a smoking history (81.6%). The median age of patients was 64 years (range: 37-77). All patients had ECOG performance status of 0 or 1. Baseline patient characteristics are summarized in **Table 1** and detailed in **Table S1**.

Clinical efficacy and safety

After neoadjuvant therapy, 39.5% (15/38) patients achieved radiological downstaging (**Fig. 2A**; **Table 2**). The ratio of patients with CR, PR, and stable disease (SD) was 5.3% (2/38), 68.4% (26/38), and 26.3% (10/38), respectively, yielding an ORR of 73.7% (28/38) (**Fig. 2B**). 91.3% (21 of 23) were recurrence-free (**Fig. 2C**). The median interval between the last dose of sintilimab and surgery was 33 days (interquartile ranges (IQRs) 5.55 days) (**Table S1**). 58.1% (18/31) patients achieved a MPR, including 12 patients with a pCR, and 41.9% (13/31) patients achieved a non-MPR (**Fig. 2D**).

For patients receiving adjuvant therapy, the median interval between the surgery and the adjuvant therapy was 32.1 days (IQRs 7.2 days) (**Table S1**). Up to January 18, 2024, the median follow-up was 14.37 months (IQRs 24.22 months) (**Table S1**). The median OS are not reached (**Fig. 2E**). 44.7% (17/38) patients experienced at least one TRAE (**Table 3**). Grade 3 TRAEs occurred only in one patient. The most common grade 1 or 2 TRAEs were nausea (15.8%). Previously unreported toxic effects were not observed, and there were no instances of discontinuation of modified neoadjuvant chemo-immunotherapy due to toxicity.

Low-dose chemotherapy combined with delayed immunotherapy has a positive effect on T lymphocytes

To investigate whether dynamic changes in peripheral blood TCR repertoire were associated with treatment efficiency during modified chemo-immunotherapy, 49 peripheral blood samples were collected from serial time-points and evaluated using TCR sequencing (**Fig. 3A**). Since the predominant number of TCR clones is an important indicator of immune response and treatment efficacy (15, 33–35), the frequency of the top 20 TCR clones was analyzed at each treatment phase. Significant increase of top 20 TCR clones were observed in phase 1 to 3 (all $P < 0.0001$; **Fig. 3B**; **Fig. S1**). An overall increase of top 20 frequency was observed in the first cycle of neoadjuvant therapy (C1). In contrast, no significant changes were observed during adjuvant therapy (**Fig. 3C**; **Table S3**). These results suggested that the peripheral blood-derived top 20 TCR clones were phased increased not only during low-dose chemotherapy, but also during immunotherapy within neoadjuvant therapy.

According to clonotype abundance, the TCR clones were divided into 3 clonotypes, including large (frequency > 0.001), median (0.001-0.0001), and small (< 0.0001) clones. A markedly increase of large clones was observed during neoadjuvant therapy (all $P < 0.05$) (**Fig. 3D** and **E**), consistent with the trend of top 20 TCR clones. No significant difference was observed in the diversity, evenness, and clonality of TCR clones across the whole treatment procedure (**Fig. 3F**; **Fig. S2; Table S4**). The changes of T lymphocytes in the tumor microenvironment across the neoadjuvant therapy were analyzed based on transcriptomic data. As shown in **Figure S3**, the CD4+ and CD8+ T lymphocytes tended to increase during neoadjuvant therapy. Collectively, the neoadjuvant treatment had a positive effect on T lymphocytes.

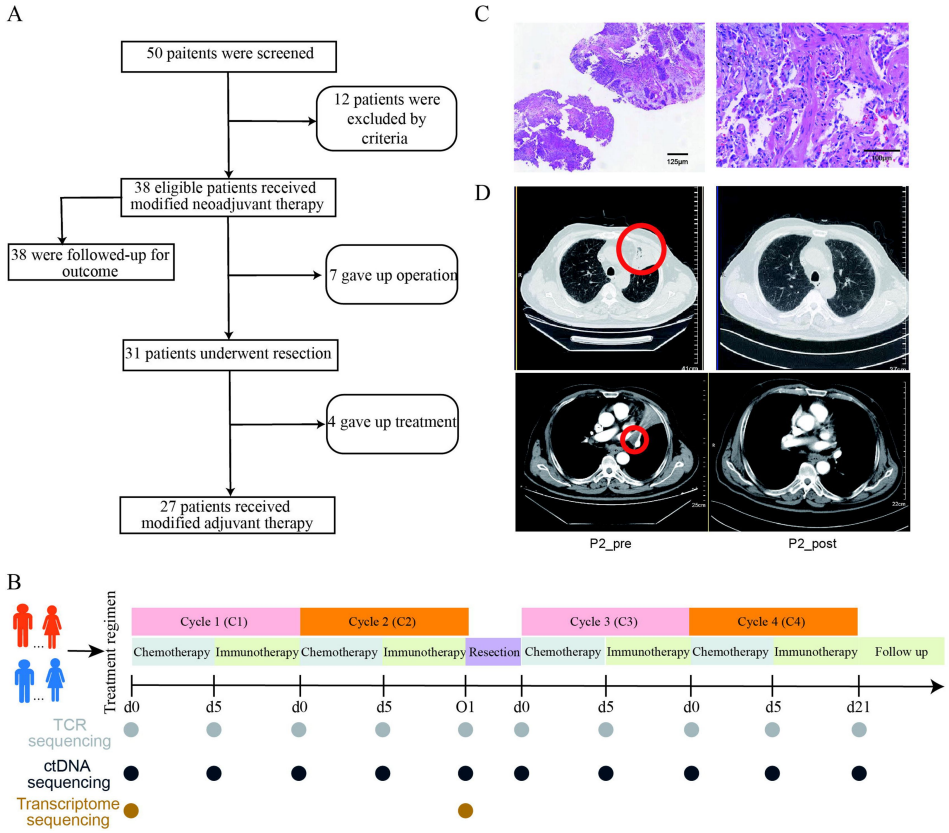

Fig. 1

Overview of study design and representative images.

(A) Flow diagram of the study. **(B)** Trial schema of the study. Patients with NSCLC received modified neoadjuvant chemo-immunotherapy, followed by surgical resection and modified adjuvant chemo-immunotherapy. Tumor tissues were collected at baseline and the surgery phase. Serial blood samples were collected at baseline and each time-point for TCR and ctDNA sequencing. **(C)** Representative hematoxylin and eosin staining of tumor specimens obtained from patient 2 (P2), who obtained pCR. **(D)** Representative computed tomographic imaging of lung lesions of P2. NSCLC, non-small cell lung cancer; TCR, T-cell receptor; ctDNA, circulating tumor DNA; pCR, pathologic complete response.

Characteristics	Overall (n=38)
Median age (range), years	64 (37-77)
Sex, n (%)	
Male	28 (78.7%)
Female	10 (26.3%)
ECOG performance status, n (%)	
0	8 (21.1%)
1	30 (78.9%)
Histological type, n (%)	
Squamous cell carcinoma	21 (55.3%)
Adenocarcinoma	17 (44.7%)
TNM stage, n (%)	
IIA	3 (7.9%)
IIB	12 (31.6%)
IIIA	23 (60.5%)
Smoking status, n (%)	
Yes	31 (81.6%)
No	7 (18.4%)
PD-L1 TPS, n (%)	
< 1%	17 (44.7%)
1-49%	13 (34.2%)
≥ 50%	8 (21.1%)

Data are presented as median (range) or n (%). Abbreviations: ECOG, Eastern Cooperative Oncology Group; TNM, tumor-nodes-metastasis; TPS, tumor proportion score.

Table 1

Baseline clinical characteristics of patients.

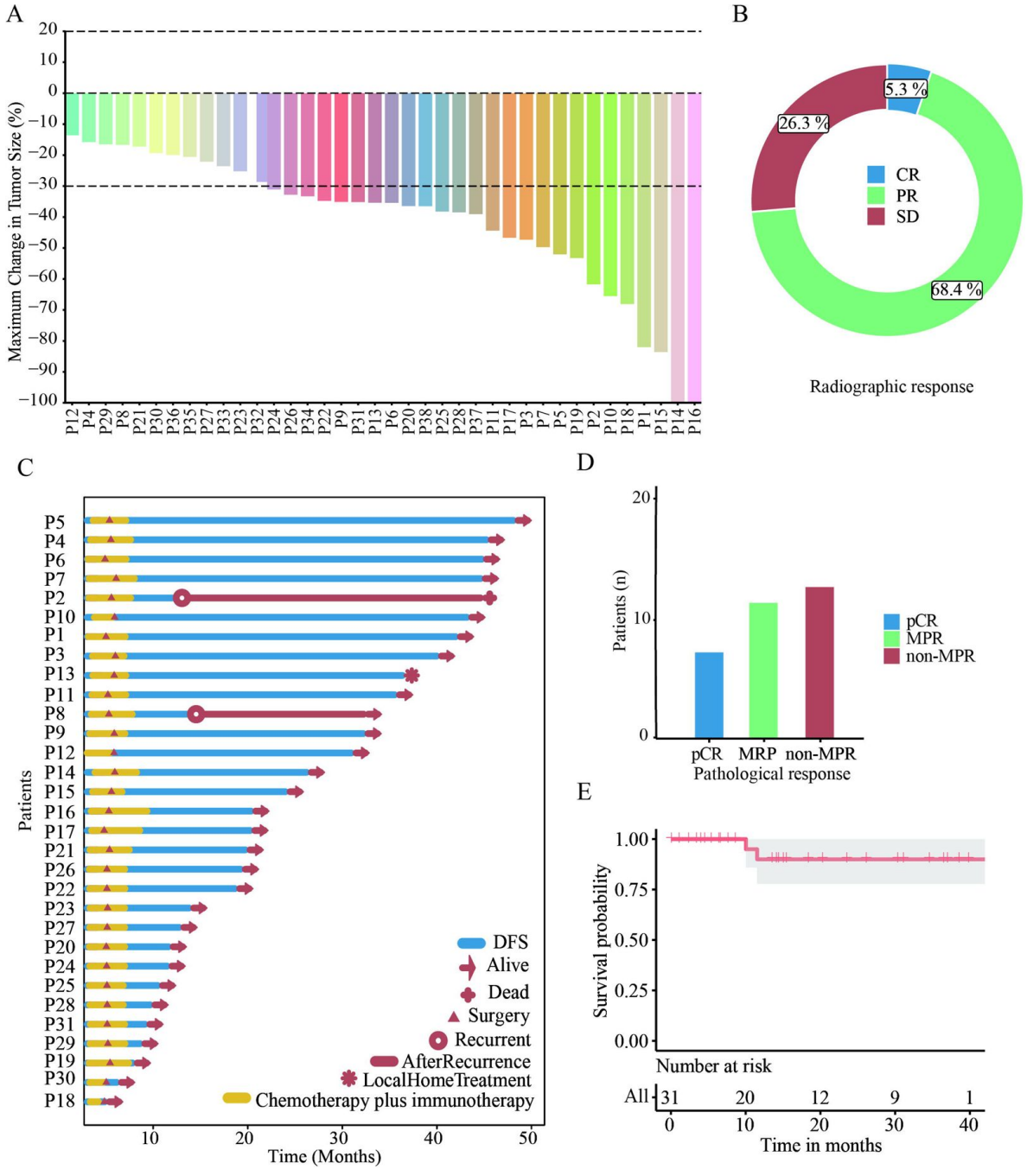

Fig. 2

Clinical efficacy of modified neoadjuvant chemo-immunotherapy.

(A) Tumor size changes from baseline based on radiological imaging. **(B)** Ring diagram showed the rate of overall response according to RECIST criteria (CR, PR, and SD) in patients. **(C)** Swimmer plot of 31 patients who underwent R0 resection involved in this trial. **(D)** Barplot showed the number of patients distributed in pathological response (pCR in blue, MPR in green, and non-MPR in red, respectively). **(E)** DFS curve for patients with modified treatment. CR, complete response; PR, partial response; SD, stable disease; pCR, completed pathological response; MPR, major pathological response; DFS, disease free survival.

Table 2

Response to modified neoadjuvant chemo-immunotherapy

Response to Treatment	Rate (%), Patient No.
Tumor down-staging rate	39.5% (15/38)
ORR	73.7% (28/38)
R0 resection rate	81.6% (31/38)
pCR rate	38.7% (12/31)
MPR rate	58.1% (18/31)

Data are presented as n%. ORR, objective response rate; pCR, pathologic complete response; MPR, major pathological response.

Treatment-related adverse events	Grade 1 or 2	Grade 3
n (%)	**16 (42.1%)**	**1 (2.6%)**
Nausea	6 (15.8%)	0
Constipation	3 (7.9%)	0
Decreased appetite	3 (7.9%)	0
Anemia	2 (5.3%)	0
Fever	2 (5.3%)	0
Rash	2 (5.3%)	0
Decreased neutrophil count	2 (5.3%)	0
Diarrhea/colitis	1 (2.6%)	0
Hepatotoxicity	1 (2.6%)	1 (2.6%)
Hypothyroidism	1 (2.6%)	0
Adrenal insufficiency	1 (2.6%)	0
Hyperthyroidism	1 (2.6%)	0
Maculopapular	0	0
Hypersensitivity	0	0
Pneumonitis	0	0
Hypophysitis	0	0
thyroiditis	0	0
Diabetes mellitus	0	0
Neutropenia	0	0

Table 3

Summary of treatment-related adverse events

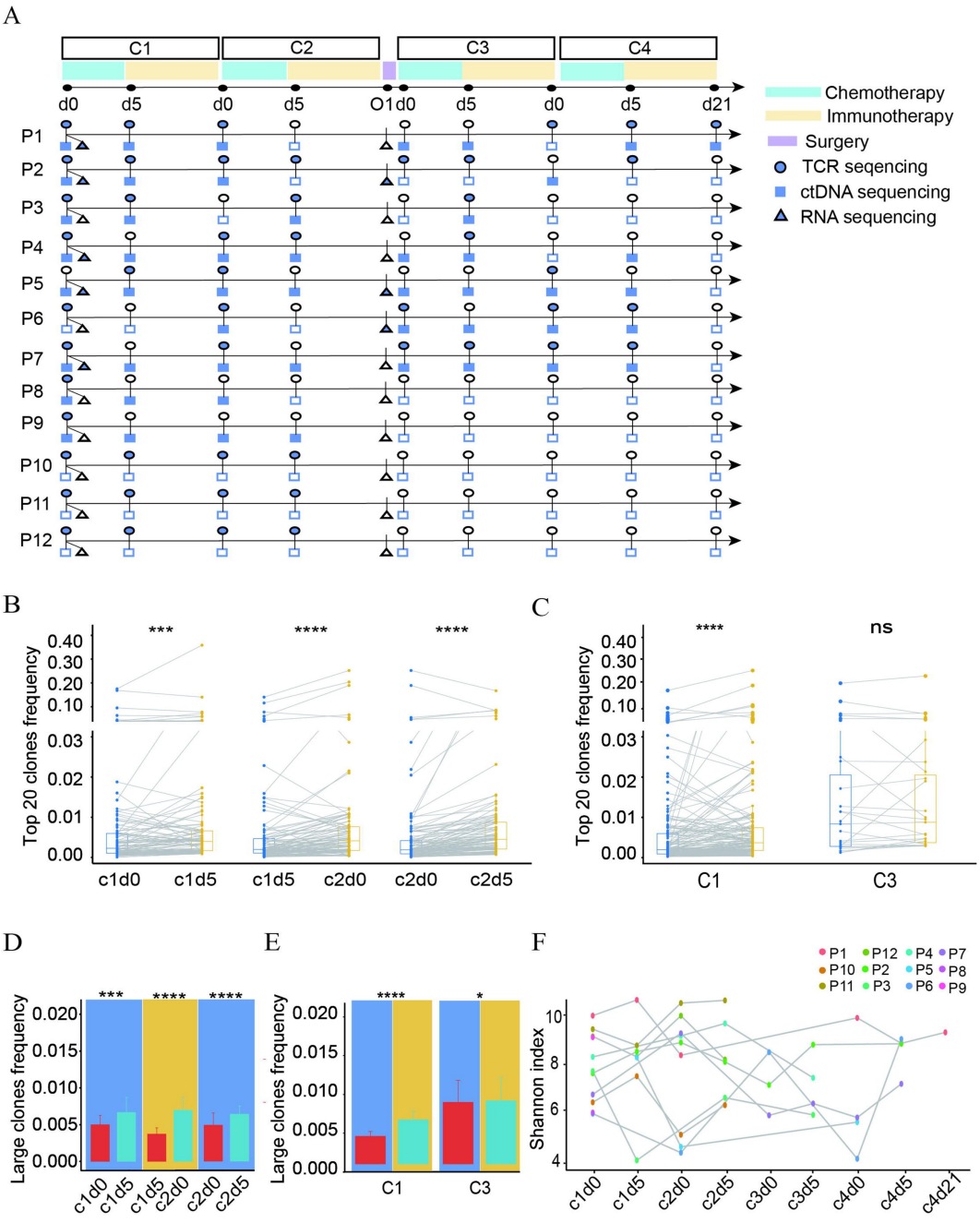

Fig. 3

Dynamic changes in T cell repertoire during therapy.

(A) The schematic of samples collection and analyses performed for each patient. Top boxes represent therapeutic phases, blue for chemotherapy, yellow for immunotherapy, and purple for operation. TCR sequencing is indicated as circle. Square and triangle represents ctDNA and RNA sequencing, respectively. Solid shapes are defined as available data, while open shapes are defined as unavailable data. **(B)** Comparison of top 20 TCR clones at different therapeutic phases. **(C)** Comparison of top 20 TCR clones at different therapeutic cycles. For boxplots, each box indicates the first quartile (Q1) and third quartile (Q3), and the black horizontal line represents the median; the upper whisker is the min[max(x), Q3 + 1.5 × IQR], and the lower whisker is the max[min(x), Q1 - 1.5 × IQR], where x represents the data, Q3 is the 75th percentile, Q1 is the 25th percentile, and IQR = Q3 - Q1. **(D)** Comparison of large TCR clones at different therapeutic phases. **(E)** Comparison of large TCR clones at different therapeutic cycles. Average and SD are shown. Wilcoxon's Signed-Rank test was used for comparison in B-E (*p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001). **(F)** Changes in diversity (Shannon index) of TCR clones for each patient. TCR, T-cell receptor; ctDNA, circulating tumor DNA; IQR, interquartile range.

Reduced tumor burden in ctDNA was observed during neoadjuvant therapy

To monitor the dynamic changes of tumor burden in ctDNA during therapy, peripheral blood samples were collected at serial time-points (T0 to T7) and evaluated via ctDNA sequencing. The landscape of genomic alternations was shown in **Figure 4A**. Among the detected genes, *TP53* (50%) was the most frequently mutated gene, followed by *ARID1A* (11%), *DNMT3A* (11%), and *PIK3CA* (11%) (**Fig. 4A**; **Table S5**). Throughout the entire treatment, the maximal somatic variant allelic frequency (maxVAF) of ctDNA had a declining trend, especially in PH1 (T0 to T1) with dramatically decreased ($P < 0.05$) (**Fig. 4B**; **Fig. S4**), indicating that low-dose chemotherapy would induce tumor cell death thus reducing tumor burden. Notably, we observed that the changes in TCR and ctDNA exhibited opposite trends during the neoadjuvant therapy (**Fig.3B** and **3D**; **Fig. 4B**). Taken together, these results suggest that low-dose chemotherapy combined with delayed immunotherapy not only cause the increase of tumor antigens shed into blood but also stimulate the body's immunity to produce more tumor antigen-specific T lymphocytes.

Discussion

In recent clinical trials, the combination of neoadjuvant chemotherapy with immunotherapy by administering them concurrently in patients with resectable NSCLC has brought about longer event-free survival (6, 8, 36). Although chemotherapy promotes tumor cell death and T lymphocyte infiltration (17, 18), it may also have negative effects on T lymphocyte proliferation (37). Therefore, in the context of combination therapy, the dosage of chemotherapy and the timing of its administration relative to the other treatment need to be better adjusted to optimize the current therapeutic efficacy.

Our previous findings from both in vitro experiments and clinical trials have suggested that administering PD-1/PD-L1 inhibitors at 1-10 days (especially 3-5 days) after chemotherapy in lung cancer patients may be more effective than administering them before or concurrently (14). In this trial, we reported the safety and efficacy of low-dose platinum-based chemotherapy combined with delayed immunotherapy for patients with stage IIA to IIIA resectable NSCLC. After modified neoadjuvant therapy, ORR was achieved in 73.7% (28/38) of patients, which was higher than the report of CheckMate-816 (54%) (6) and comparable to those of NADIM (76%) (8) and LungMate 002 (76%) (38) trials. MPR was achieved in 47.4% (18/38) patients, with 31.6% (12/38) achieving pCR. During a median follow-up of 14.37 months, 91.3% (21/23) patients were recurrence-free. These results indicated that modified neoadjuvant chemo-immunotherapy was effective in patients with resectable NSCLC. Only 2.6% (1/38) patients experienced serious TRAEs after receiving modified neoadjuvant treatment, which is lower than the rates reported in CheckMate 816 (33.5%), NAMID (30.0%) (8), and LungMate 002 (6.0%) (38) trials. The reduction of chemotherapeutics may contribute to the low incidence of serious TRAEs. Additionally, we did not observe treatment discontinuation due to toxicity. These results provide evidences that low-dose platinum-based chemotherapy combined with delayed immunotherapy as a neoadjuvant therapy is safe and less toxic.

Previous research has illustrated an association between clinical benefit and TCR repertoire as well as ctDNA level at baseline (39, 40). However, the dynamic changes in the TCR repertoire and ctDNA level during neoadjuvant treatment have been less studied. In our study, we found that the predominant TCR clones were greatly increased after the treatment of chemotherapy from phase 1 to 3, indicating that low-dose platinum-based chemotherapy did not inhibit the proliferation of predominant T cell clones which create a beneficial microenvironment for subsequent immunotherapy. Moreover, ICI treatment significantly increased the number of

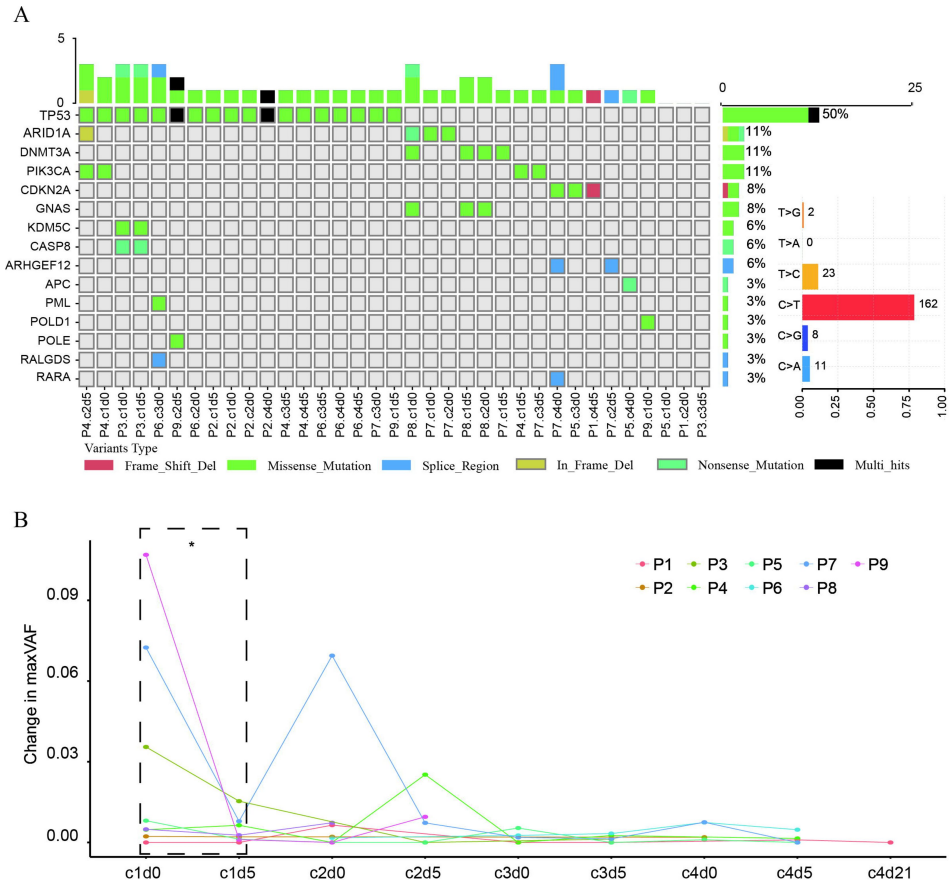

Fig. 4

Characterization of ctDNA and the alteration of tumor burden during treatment.

(A) Genomic landscape of mutations detected in plasma via ctDNA sequencing. Frequency of mutated genes were shown on the bar of middle and the proportion was shown in numerical form. Counts of mutations for each sample were displayed on top bar. Frequency for each of the six substitutions of the bases spectrum was shown on right. The scale lines below show the proportion of various mutation substitutions. **(B)** Changes in maxVAF across the entire course of therapy. Wilcoxon's Signed-Rank test was used for comparison. (*$p < 0.05$). ctDNA, circulating tumor DNA; maxVAF, maximal somatic variant allelic frequency.

predominant TCR clones during neoadjuvant therapy, suggesting that immunotherapy has a positive impact on TCR clones, consistent with the study by Forde et al (41). During adjuvant therapy, no significant fluctuation in the predominant TCR clones was observed, probably because the tumor lesions were cleared. Under such circumstances, no tumor antigen was released, thus no tumor antigen-specific T cells were proliferated. Similarly, no significant change was observed in evenness or clonality of TCR. MaxVAF of ctDNA was dramatically dropped during phase 1, which is accordance with previous studies (42–44). Notably, the maxVAF of ctDNA and predominant TCR clones showed opposite trends of change in phase 1. Moreover, the maxVAF of ctDNA was close to 0 during adjuvant therapy stage.

Our study had several limitations that need to be acknowledged. First, it was a pilot study with a relatively small cohort and lacked a control arm. Therefore, validation of our findings in larger cohorts is necessary. Second, the follow-up period was short, and longer-term results are needed to investigate whether modified therapy provides long-term survival benefits. Finally, innovative techniques should be incorporated to further explore the underlying mechanisms of superior clinical efficacy and reduced TRAEs.

In this study, the modified neoadjuvant low-dose platinum-based chemotherapy combined with delayed immunotherapy in patients with resectable NSCLC exhibited promising efficacy and feasibility. The multi-omics analyses provide the dynamic trajectories of the TCR repertoire and tumor burden, which can aim in better understanding the mechanisms of superior efficiency and the feasibility of modified chemo-immunotherapy. In the future, it is possible to conduct large-scale clinical research using this modified chemo-immunotherapy.

Data availability statement

All the analysis data can be found in the supplementary tables. The dataset supporting the conclusions of this article is available in the Genome Sequence Archive for Human (GSA-H) repository with accession number (HRA002904) and can be accessed thru the URL (BIG Search - National Genomics Data Center - BIG Search (cncb.ac.cn)) after reasonable request.

Acknowledgements

We would like to thank Innovent Biologics, Inc for providing sintilimab for this study.

Additional information

Declarations

Ethics approval and consent to participate

This study was conducted according to the guidelines of the Ethics Committee of the General Hospital of the People's Liberation Army (S2020-144-01) and written informed consent was obtained from all participants.

Author's contributions

C.Y. Liang: Conceptualization, Formal analysis, Methodology, Writing - original draft. Q. Song: Formal analysis, Methodology, Funding acquisition, Writing - original draft. W.H. Zhou and N. Li: Formal analysis, Methodology, Writing - original draft. C.H. Pan, Q. Xiong B. Yang, and X.L Zhang: Formal analysis. S.H Zhao, W.W. Shi, X. Yan, J.T. Guo, S.J. Sun, Z.H. Dong, and Y.P. Long: Validation, Writing - reviewing & editing. B. Yang, J. Li, Y. Hu, and H.T. Luo: Conceptualization, Investigation, Validation, Verification of underlying data, Supervision, Writing - reviewing & editing.

Funding

This study was supported by the National Natural Science Fund of the People's Republic of China (NSFC) No.81902912.

Additional files

Supplemental figures

Supplementary Table 1. *Clinical characteristics of patients.*

Supplemental Table 2. *List of treatment regimens.*

Supplemental Table 3. *List of top 20 TCR clones frequency across the whole modified therapy.*

Supplemental Table 4. *TCR characteristics across the whole modified therapy.*

Supplemental Table 5. *T cells abundance estimated by xCell.*

Supplementary Table 6. *T cells abundance estimated by ImmunCellAI.*

Supplementary Table 7. *Annotation of ctDNA somatic mutations.*

Note

This reviewed preprint has been updated to correct a corresponding email address.

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Reviewer #1 (Public review):

Liang et al. have conducted a small-scale pilot study focusing on the feasibility and tolerability of Low-dose chemotherapy combined with delayed immunotherapy in the neoadjuvant treatment of non-small cell lung cancer. The design of delayed immunotherapy after chemotherapy is relatively novel, while the reduced chemotherapy, although somewhat lacking in innovation, still serves as an early clue for exploring future feasible strategies. Also, the dynamic ctDNA and TCR profiles could give some important hints of intrinsic tumor reaction.

However, as the author mentioned in the limitation part, due to the small sample size and lack of a control group, we cannot fully understand the advantages and disadvantages of this approach compared to standard treatment. Compared to standard immunotherapy, the treatment group in this study has three differences: (1) reduced chemotherapy, (2) the use of cisplatin instead of the commonly used carboplatin in neoadjuvant therapy trials, and (3) delayed immunotherapy. Generally, in the exploration of updated treatment strategies, the design should follow the principle of "controlling variables." If there are too many differences at once, it becomes difficult to determine which variable is responsible for the effects, leading

to confusion in the interpretation of the results. Moreover, the therapeutic strategy may lack practical clinical operability due to the long treatment duration.

Furthermore, in the exploration of biomarkers, the authors emphasized the procedure of whole RNA sequencing in tumor tissues in the method section, and this was also noted in the flowchart in Figure 1. However, I didn't find any mention of RNA-related analyses in the Results section, which raises some concerns about the quality of this paper for me. If the authors have inadvertently omitted some results, they should supplement the RNA-related analyses so that I can re-evaluate the paper.

To sum up, this article exhibited a certain degree of innovation to some extent, However, due to its intrinsic design defects and data omissions, the quality of the research warranted further improvement.

https://doi.org/10.7554/eLife.99720.2.sa2

Reviewer #2 (Public review):

Summary:

In this single center, single arm, open label non-randomised study the authors tested the use of paclitaxel at 180-220 mg/m2 and cisplatin at 60mg/m2 in patients with squamous NSCLC and pemetrexed at 500mg/m2 and cisplatin at 60mg/m2 in adenocarcinoma of lung origin in the neoadjuvant setting. The chemotherapy appears to have been given at a relatively standard dose; though the platin dose at 60mg/m2 is somewhat lower than has been used in the checkmate 816 trial (75mg/m2/dose), this is a well-established dose for NSCLC.

Key differences to currently approved neoadjuvant chemo-ICI treatment is that anti-PD1 antibody sintilimab (at 200mg/dose) was given on day 5 and that only 2 cycles of chemotherapy were given pre surgery, but then repeated on two occasions post surgery. Between May/2020 and Nov/2023 50 patients were screened, 38 went on to have this schedule of tx, 31 (~82%) went on to have surgery and 27 had the adjuvant treatment. The rate of surgery is entirely consistent with the checkmate 816 data.

Question to the authors:

It would be very helpful to understand why 7 (~18% of the population) patients did not make it to surgery and whether this is related to disease progression, toxicity or other reasons for withdrawal.

The key clinical endpoints were pCR and mPR rates. 2/38 patients are reported to have achieved a radiological pCR but only 31 patients underwent surgery with histological verification. Supp table2 suggests that 10/31 patients achieved a pCR, 6/31 additional patients achieved a major pathological response and that 13/31 did not achieve a major pathological response

It would be really helpful for understanding the clinical outcome to present the histopathological findings in the text in a bit more detail and to refer the outcome to the radiological findings. I note that the reference for pathological responses incorrectly is 38 patients as only 31 patients underwent surgery and were evaluated histologically.

The treatment was very well tolerated with only 1 grade 3 AE reported. The longer term outcome will need to be assessed over time as the cohort is very 'young'. It is not clear what the adjuvant chemo-ICI treatment would add and how this extra treatment would be evaluated for benefit - if all the benefit is in the neoadjuvant treatment then the extra post-operative tx would only add toxicity

Please consider what the two post-operative chemo-ICI cycles might add to the outcome and how the value of these cycles would be assessed. Would there be a case for a randomised assessment in the patients who have NOT achieved a mPR histologically?

While the clinical dataset identifies that the proposed reduced chemo-ICI therapy has clinical merit and should be assessed in a randomized study, the translational work is less informative.

The authors suggest that the treatment has a positive impact on T lymphocytes. Blood sampling was done at day 0 and day 5 of each of the four cycle of chemotherapy with an additional sample post cycle 4. The authors state that data were analysed at each stage.

The data in Figure 3B are reported for three sets of pairs: baseline to pre day 5 in cycle 1, day 5 to day 21 in cycle 1, baseline of cycle to to day 5. It remains unclear whether the datasets contain the same top 20 clones and it would be very helpful to show kinetic change for the individual 'top 20 clones' throughout the events in individual patients; as it stands the 'top20 clones' may vary widely from timepoint to timepoint. Of note, the figures do not demonstrate that the top 20 TCR clones were 'continuously increased'.

Instead, the data suggest that there are fluctuations in the relative distributions over time but that may simply be a reflection of shifts in T cell populations following chemotherapy rather than of immunological effects in the cancer tissue.
Consistent with this the authors conclude (line 304/5): "No significant difference was observed in the diversity, evenness, and clonality of TCR clones across the whole treatment procedure" and this seems to be a more persuasive conclusion than the statement 'that a positive effect on T lymphocytes was observed' - where it is also not clear what 'positive' means.

The text needs a more balanced representation of the data: only a small subset of four patients appear to have been evaluated to generate the data for figure 3B and only three patients (P5, P6, P7) can have contributed to figure 3C if the sample collection is represented accurately in Figure 3A.

The text refers to flow cytometric results in SF3. However, no information is given on the flow cytometry in M&M, markers or gating strategy.

Please consider changing the terminology of the 'phases' into something that is easier to understand. One option would be to use a reference to a more standard unit (cycle 1-4 of chemotherapy and then d0/d5/d21).

Please make it explicit in the text that molecular analyses were undertaken for some patients only, and how many patients contribute to the data in figures 3B-F. Figure 3A suggests paired mRNA data were obtained in 2 patients (P2 and P5) but I cannot find the results on these analyses; four individual blood samples to assess TCR changes int PH1/PH2/PH3and PH4 were only available in four patients (P4,P5,P7,P9). Only three patients seem to have the right samples collected to allow the analysis for 'C3' in figure 3C.

Please display for each of the 'top 20 clones' at any one timepoint how these clones evolve throughout the study; I expect that a clone that is 'top 20' at a given timepoint may not be among the 'top twenty' at all timepoints.

Please also assess if the expanded clonotypes are present (and expanded) in the cancer tissue at resection, to link the effect in blood to the tumour. Given that tissue was collected for 31 patients, mRNA sequencing to generate TCR data should be possible to add to the blood analyses in the 12 patients in Figure 3A. Without this data no clear link can be made to events in the cancer.

Please provide in M&M the missing information on the flow cytometry methodology (instrument, antibody clones, gating strategy) and what markers were used to define T cell subsets (naïve, memory, central memory, effector memory).

The authors also describe that ctDNA reduces after chemo-ICI treatment. This is well documented in their data but ultimately irrelevant: if the cancer volume is reduced to the degree of a radiological or pathological response /complete response then the quantity of circulating DNA from the cancer cells must reduce. More interesting would be the question whether early changes predict clinical outcome and whether recurrent ct DNA elevations herald recurrence.

Please probe whether the molecular data identify good radiological or pathological outcomes before cycle 2 is started and whether the ctDNA levels identify patients who will have a poor response and/or who relapse early.

https://doi.org/10.7554/eLife.99720.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Liang et al. have conducted a small-scale pilot study focusing on the feasibility and tolerability of Low-dose chemotherapy combined with delayed immunotherapy in the neoadjuvant treatment of non-small cell lung cancer. The design of delayed immunotherapy after chemotherapy is relatively novel, while the reduced chemotherapy, although somewhat lacking in innovation, still serves as an early clue for exploring future feasible strategies. Also, the dynamic ctDNA and TCR profiles could give some important hints of intrinsic tumor reaction.*
>
> *However, as the author mentioned in the limitation part, due to the small sample size and lack of a control group, we cannot fully understand the advantages and disadvantages of this approach compared to standard treatment. Compared to standard immunotherapy, the treatment group in this study has three differences: (1) reduced chemotherapy, (2) the use of cisplatin instead of the commonly used carboplatin in neoadjuvant therapy trials, and (3) delayed immunotherapy. Generally, in the exploration of updated treatment strategies, the design should follow the principle of "controlling variables." If there are too many differences at once, it becomes difficult to determine which variable is responsible for the effects, leading to confusion in the interpretation of the results. Moreover, the therapeutic strategy may lack practical clinical operability due to the long treatment duration.*

Thank you for your advice. As you pointed out, incorporating too many variables can obscure research findings. Our study focuses on two primary objectives: (1) to demonstrate that our approach is less toxic than the standard regimen; and (2) to fully activate the immune system in order to achieve better therapeutic outcomes. Based on these two objectives, we reduced chemotherapy dosage to alleviate toxicity, and perform delayed immunotherapy administration to alleviate the killing of activated immune cells by chemotherapy so as to maximize the immune response. Therefore, the two variables of reduced chemotherapy and delayed immunotherapy are unified in this study. The reduction of cisplatin to 60mg/m2 is supported by data for Chinese people; A retrospective study conducted by our center found

that delayed immunotherapy also has great therapeutic effects. Considering the previous blood toxicity of carboplatin and albumin paclitaxel, we replaced carboplatin with cisplatin to alleviate bone marrow suppression. Usually, our patients are hospitalized for 4-7 days to receive treatment, observe and manage potential side effects, including nausea, vomiting, diarrhea, bone marrow suppression and so on. Therefore, it is convenient and feasible for immunotherapy administration on the 5th day.

> *Furthermore, in the exploration of biomarkers, the authors emphasized the procedure of whole RNA sequencing in tumor tissues in the method section, and this was also noted in the flowchart in Figure 1. However, I didn't find any mention of RNA-related analyses in the Results section, which raises some concerns about the quality of this paper for me. If the authors have inadvertently omitted some results, they should supplement the RNA-related analyses so that I can re-evaluate the paper.*

Thanks for your comment. In this study, we employed a multi-omics approach involving whole transcriptome, ctDNA, and TCR sequencing to investigate the effects of a neoadjuvant treatment on NSCLC. The sequencing details are described in the Materials and Methods section. RNA-related analyses are presented in Figure S3. Given that our primary focus is on the impact of this modified treatment on immune cells, we estimate immune cell compositions by using the xCell and immunCellAI algorithms based on the RNA sequencing results. The estimated immune cell profiles have been added to Supplementary Tables 5 and 6.

> *To sum up, this article exhibited a certain degree of innovation to some extent, However, due to its intrinsic design defects and data omissions, the quality of the research warranted further improvement.*

Thanks for your comment. We have provided a more detailed explanation of the administration for all patients. Additionally, we have clarified and supplemented the sequencing results to enhance the clarity and overall quality of the article.

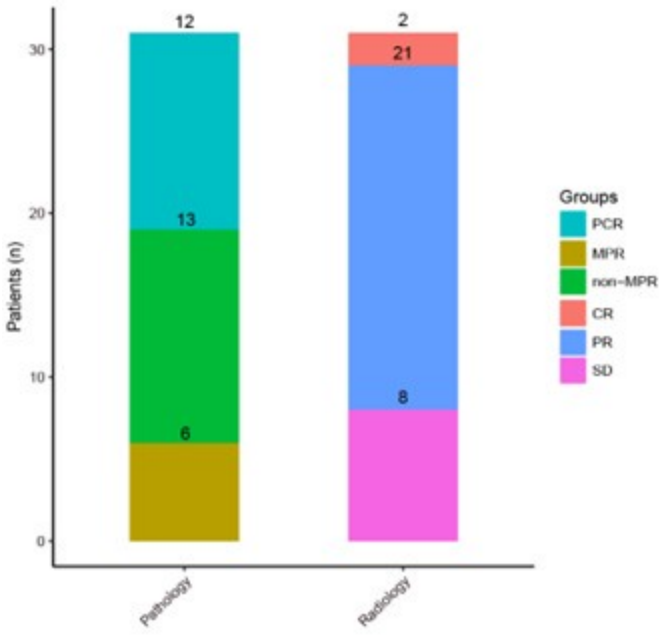
> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *In this single center, single arm, open label non-randomised study the authors tested the use of paclitaxel at 180-220 mg/m2 and cisplatin at 60mg/m2 in patients with squamous NSCLC and pemetrexed at 500mg/m2 and cisplatin at 60mg/m2 in adenocarcinoma of lung origin in the neoadjuvant setting. The chemotherapy appears to have been given at a relatively standard dose; though the platin dose at 60mg/m2 is somewhat lower than has been used in the checkmate 816 trial (75mg/m2/dose), this is a well-established dose for NSCLC.*
>
> *Key differences to currently approved neoadjuvant chemo-ICI treatment is that anti-PD1 antibody sintilimab (at 200mg/dose) was given on day 5 and that only 2 cycles of chemotherapy were given pre surgery, but then repeated on two occasions post surgery. Between May/2020 and Nov/2023 50 patients were screened, 38 went on to have this schedule of tx, 31 (~82%) went on to have surgery and 27 had the adjuvant treatment. The rate of surgery is entirely consistent with the checkmate 816 data.*
>
> *Question to the authors:*
>
> *It would be very helpful to understand why 7 (~18% of the population) patients did not make it to surgery and whether this is related to disease progression, toxicity or other reasons for withdrawal.*

Thank you for your comment. No patients were denied surgery due to disease progression or side effects. 7 patients did not undergo surgery: three declined to undergo total pneumonectomy, 2 were unable to come to our hospital for treatment because of the COVID-19 pandemic, and 2 were ineligible for radical surgery due to tumor invasion of the arteries.

> *The key clinical endpoints were pCR and mPR rates. 2/38 patients are reported to have achieved a radiological pCR but only 31 patients underwent surgery with histological verification. Supp table2 suggests that 10/31 patients achieved a pCR, 6/31 additional patients achieved a major pathological response and that 13/31 did not achieve a major pathological response.*
>
> *It would be really helpful for understanding the clinical outcome to present the histopathological findings in the text in a bit more detail and to refer the outcome to the radiological findings. I note that the reference for pathological responses incorrectly is 38 patients as only 31 patients underwent surgery and were evaluated histologically.*

Thanks for your comment. The ITT population consisted of 38 individuals, of whom 31 underwent surgery. After surgery, 18 patients achieved MPR, including 12 achieved pCR and 13 achieved non-MPR. So for ITT population, the rate of pCR and MPR is 12/38 (31.6%) and 18/38 (47.4%) respectively; for patients who have completed surgery, both pCR and MPR have improved, accounting for 12/31 (38.7%) and 18/31 (58.1%) respectively (Results, line 268 to 269).

Author response image 1.



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>
> *Please consider what the two post-operative chemo-ICI cycles might add to the outcome and how the value of these cycles would be assessed. Would there be a case for a*

> *randomised assessment in the patients who have NOT achieved a mPR histologically?*

Thanks for your comment. The purpose of postoperative adjuvant therapy is to prevent recurrence and metastasis. Both clinical trial Keynote091 and Impower010 have achieved positive test results. The clinical trial design of Checkmate-77T is neoadjuvant therapy followed by surgery and adjuvant therapy. Checkmate-77T resulted in significantly longer event-free survival than chemotherapy in patients with resectable NSCLC. So we designed this perioperative treatment method, which is currently a common approach, hoping to reduce tumor burden and improve surgical remission rate through neoadjuvant therapy; and to kill residual tumor cells and prolong the DFS through adjuvant therapy. As for DFS, follow-up shows that there are currently 3 cases of recurrence, but the overall data is not yet mature (updated in Table S1). The side effect includes all patients who received neoadjuvant therapy and adjuvant therapy, and the addition of immunotherapy shows no new safety signals.

> *While the clinical dataset identifies that the proposed reduced chemo-ICI therapy has clinical merit and should be assessed in a randomized study, the translational work is less informative.*

Thanks for your comment. As mentioned in the shortcomings of the article, our research is preliminary and exploratory, and more large-scale randomized studies are needed to be invested in the future.

> *The authors suggest that the treatment has a positive impact on T lymphocytes. Blood sampling was done at day 0 and day 5 of each of the four cycle of chemotherapy with an additional sample post cycle 4. The authors state that data were analysed at each stage.*
>
> *The data in Figure 3B are reported for three sets of pairs: baseline to pre day 5 in cycle 1, day 5 to day 21 in cycle 1, baseline of cycle to to day 5. It remains unclear whether the datasets contain the same top 20 clones and it would be very helpful to show kinetic change for the individual 'top 20 clones' throughout the events in individual patients; as it stands the 'top20 clones' may vary widely from timepoint to timepoint. Of note, the figures do not demonstrate that the top 20 TCR clones were 'continuously increased'.*

Thanks for your comment. The data in Fig. 3B do not represent the overlapping top 20 clones across all samples but rather illustrate the changes in the individual top 20 clones for each patient. The changes in the top 20 TCR clones during neoadjuvant treatment for specific samples are shown in Fig. S1. Due to tumor heterogeneity, both within and between samples, the top 20 clones for each patient at the same time point may differ. Additionally, since the top 20 TCR clones can vary between stages as a result of antigen exposure over time, the top 20 clones for the same patient may also differ across different time points. Indeed, when analyzing the data, we measured the dynamic changes of the top 20 TCR clones across three stages in cycle 1, and describing these changes as "continuously increased" may not be entirely accurate. Therefore, we believe it is more accurate to correct it to a phased increase. (Results line 293).

> *Instead, the data suggest that there are fluctuations in the relative distributions over time but that may simply be a reflection of shifts in T cell populations following chemotherapy rather than of immunological effects in the cancer tissue.*
> *Consistent with this the authors conclude (line 304/5): "No significant difference was observed in the diversity, evenness, and clonality of TCR clones across the whole treatment procedure" and this seems to be a more persuasive conclusion than the statement 'that a positive effect on T lymphocytes was observed' - where it is also not clear what 'positive' means.*

Thanks for your comment. The scores for diversity, evenness, and clonality assess changes in the overall TCR repertoire. In our cohort, we did not observe significant changes in these three metrics throughout the treatment process, indicating the overall stability of the TCR repertoire. Despite this overall stability, we observed a significant increase in the top 20 and large clones—representative of major TCR clone dynamics—during the treatment period. Additionally, integrating RNA results (Table S5-S6 and Fig. S3) from baseline and surgical samples, we found an increasing trend in the proportion of T cells following neoadjuvant therapy. Therefore, we suggested that the treatment has a positive effect on T lymphocytes.

> *The text needs a more balanced representation of the data: only a small subset of four patients appear to have been evaluated to generate the data for figure 3B and only three patients (P5, P6, P7) can have contributed to figure 3C if the sample collection is represented accurately in Figure 3A.*

Thanks for your comment. In Fig. 3B, we utilized TCR data from six patients (P1, P2, P3, P10, P11, P12) for the period from day 1 to day 5 of cycle 1. For the period from day 5 of cycle 1 to day 1 of cycle 2, we used data from six patients (P1, P2, P5, P10, P11, P12). For the period from day 1 of cycle 2 to day 5 of cycle 2, we included data from five patients (P2, P4, P10, P11, P12). In Fig. 3C, we used TCR data from eight patients (P1, P2, P4, P6, P7, P10, P11, P12) to generate the images for cycle 1, and data from two patients (P6, P7) to create the images for cycle 3. Therefore, the sampling illustration in Fig. 3A is accurate.

> *The text refers to flow cytometric results in SF3. However, no information is given on the flow cytometry in M&M, markers or gating strategy.*

Thanks for your comment. In this study, we performed tissue sampling and whole transcriptome sequencing at both the baseline and surgical stages. Based on the sequencing results, we evaluated T cell populations using two algorithms, xCell and immunoCellAI, and detailed the analysis procedures in the Methods and Materials section. Additionally, we have included the assessment results from both algorithms in Supplementary Tables 5 and 6.

> *Please consider changing the terminology of the 'phases' into something that is easier to understand. One option would be to use a reference to a more standard unit (cycle 1-4 of chemotherapy and then d0/d5/d21).*

Thanks for your advice. Since each treatment cycle consists of both chemotherapy and immunotherapy, with chemotherapy administered on day 1 and immunotherapy on day 5 of each cycle, blood samples are collected at these two time points. Following your suggestion, we will use the notation d0/d5 within each treatment cycle to better clarify this process for the readers.

> *Please make it explicit in the text that molecular analyses were undertaken for some patients only, and how many patients contribute to the data in figures 3B-F. Figure 3A suggests paired mRNA data were obtained in 2 patients (P2 and P5) but I cannot find the results on these analyses; four individual blood samples to assess TCR changes int PH1/PH2/PH3and PH4 were only available in four patients (P4,P5,P7,P9). Only three patients seem to have the right samples collected to allow the analysis for 'C3' in figure 3C.*

Thanks for your comment. In Fig. 3B and 3D, we used TCR data from six patients (P1, P2, P3, P10, P11, P12) for the period from day 0 to day 5 of cycle 1. For the period from day 5 of cycle 1 to day 0 of cycle 2, data from six patients (P1, P2, P5, P10, P11, P12) were used. For the period from day 0 of cycle 2 to day 5 of cycle 2, we included data from five patients (P2, P4,

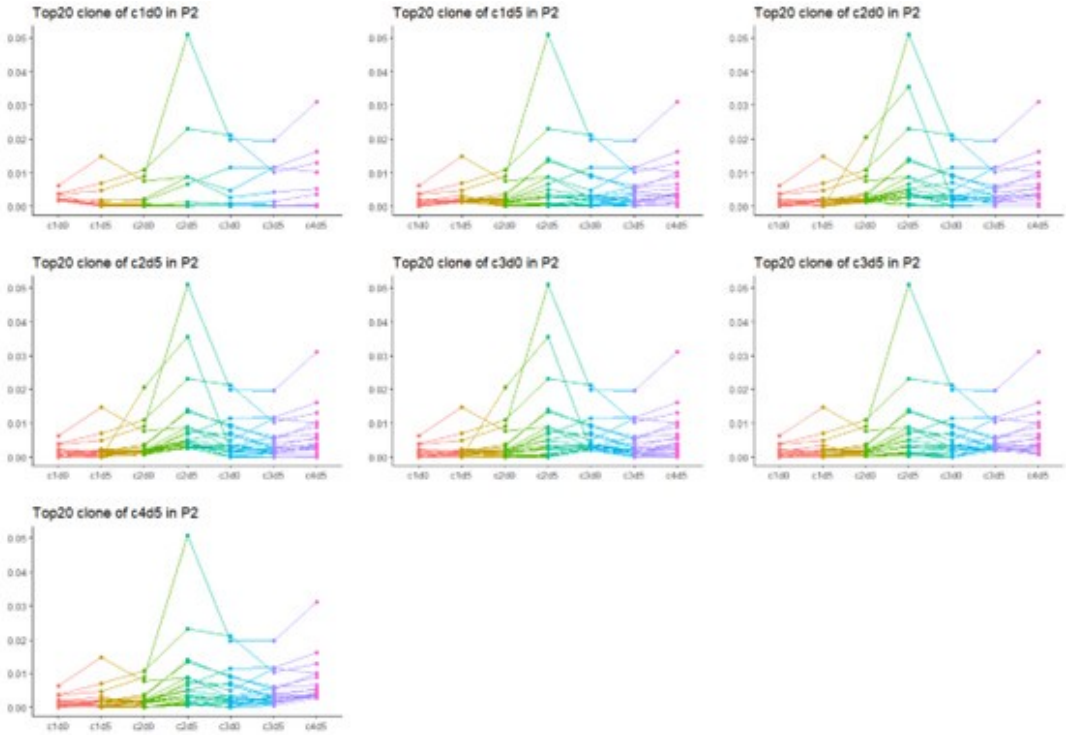
P10, P11, P12). In Fig. 3C and 3E, TCR data from eight patients (P1, P2, P4, P6, P7, P10, P11, P12) were used to generate the images for cycle 1, while data from two patients (P6, P7) were used to create the images for cycle 3. In Fig. 3F, all patients who underwent sequencing are included in the analysis, with each patient's data represented by dots of different colors.

For the mRNA data, we sampled and sequenced five patients (P1, P2, P4, P5, P7) before treatment. During the surgical phase, we sampled and sequenced three patients (P2, P5, P6). The T cell assessments and comparisons based on the mRNA sequencing results are presented in Fig. S3 and Tables S5-S6.

> *Please display for each of the 'top 20 clones' at any one timepoint how these clones evolve throughout the study; I expect that a clone that is 'top 20' at a given timepoint may not be among the 'top twenty' at all timepoints.*

Thanks for your comment. Yes, due to the heterogeneity of tumors, a variety of different antigens are exposed during the course of cancer treatment. As a result, the formation of TCR dominant clones is a dynamic process, with new dominant clones emerging at each stage. Therefore, the top 20 clones at each time point do not necessarily represent the overall top 20 clones across all time points. However, there is still some overlap in the dominant TCR clones. We have chosen to present the data from P2, which provides the most complete results throughout the entire treatment process.

Author response image 2.



> *Please also assess if the expanded clonotypes are present (and expanded) in the cancer tissue at resection, to link the effect in blood to the tumour. Given that tissue was collected for 31 patients, mRNA sequencing to generate TCR data should be possible to add to the blood analyses in the 12 patients in Figure 3A. Without this data no clear link can be made to events in the cancer.*

Thanks for your comment. Due to limitations in sampling conditions, we were unable to collect samples from all patients at every time point. As shown in Fig. 3A, we performed tissue sampling and RNA sequencing on five patients (P1, P2, P4, P5, P7) before treatment. During the surgical phase, we sampled and conducted RNA sequencing on three patients (P2, P5, P6). This study primarily focuses on TCR analysis in peripheral blood. The relationship between peripheral blood TCR and tissue TCR clones will be addressed in future research.

> *Please provide in M&M the missing information on the flow cytometry methodology (instrument, antibody clones, gating strategy) and what markers were used to define T cell subsets (naïve, memory, central memory, effector memory).*

Thanks for your comment. In this study, we evaluated immune cells based on RNA sequencing results rather than using flow cytometry. Subsequently, we compared T cell subsets between the baseline and post-neoadjuvant treatment stages. The steps for RNA sequencing and the evaluation of immune cells using the xCell and ImmunoCellAI algorithms are detailed in the Methods and Materials section. The comparison of T cell subsets is presented in Fig. S3. The estimated immune cell data have been added to Tables S5 and S6.

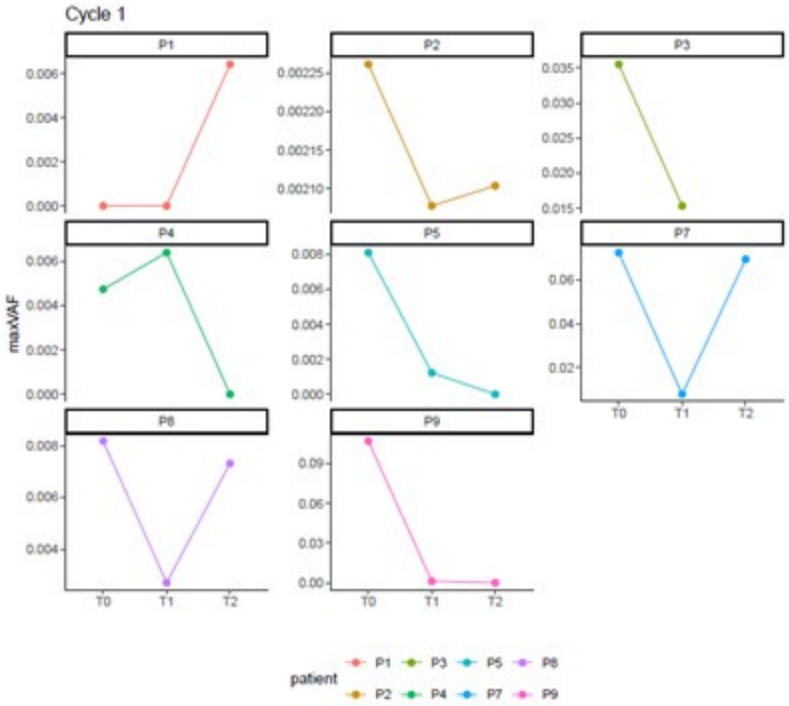
> *The authors also describe that ctDNA reduces after chemo-ICI treatment. This is well documented in their data but ultimately irrelevant: if the cancer volume is reduced to the degree of a radiological or pathological response /complete response then the quantity of circulating DNA from the cancer cells must reduce. More interesting would be the question whether early changes predict clinical outcome and whether recurrent ct DNA elevations herald recurrence.*

Thanks for your comment. If the tumor responds to treatment, its volume will decrease. Over the long term, ctDNA levels in the blood are expected to decline. However, in the short term, as tumor cells are killed, there may be a surge of ctDNA released into the patient's bloodstream, potentially causing a rise in the maxVAF. Based on the current follow-up data, the ctDNA maxVAF for patient P8 has increased compared to baseline levels. However, given the relatively short follow-up period, no recurrence has been observed yet.

> *Please probe whether the molecular data identify good radiological or pathological outcomes before cycle 2 is started and whether the ctDNA levels identify patients who will have a poor response and/or who relapse early.*

Thanks for your comment. Before initiating Cycle 2 of treatment, we observed all patients whom performed ctDNA sequencing. Among them, Patients P1 to P4 were classified as MPR, whereas Patients P5 to P9 were categorized as non-MPR. It was noted that Patients P7 and P8 showed a trend of increasing maximum variant allele frequency (maxVAF) in their ctDNA. Thus, 50% (2 out of 4) of the MPR patients could be identified as having potential issues through molecular testing before Cycle 2. Additionally, only P3 experienced a recurrence, which was predicted by molecular testing prior to starting cycle 2.

Author response image 3.



> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *I have some detailed comments for the authors:*
>
> *(1) Please explain the reason for putting forward the opinion that "cytotoxic drugs with standard doses and anti-PD1 antibody were administrated on the same day (9), which may result in unsatisfactory eradication rates and relatively high incidence of severe treatment-related adverse events (TRAEs)" (Page 3 Line 76), especially "unsatisfactory eradication rates". Is this based on actual evidence, or is it purely theoretical speculation?*

Thanks for your comment. Our team have done relative research to explore impact of the combined timing of PD-1/PD-L1 inhibitors and chemotherapy on the outcomes in patients with refractory lung cancer. Our findings suggest that administering PD-1/PD-L1 inhibitors 1-10 days (especially 3-5 days) after chemotherapy is superior to administering PD-1/PD-L1 inhibitors before or concurrent with chemotherapy in patients with refractory lung cancer, but this result needs to be further explored by prospective studies. So we infer that cytotoxic drugs with standard doses and anti-PD1 antibody were administrated on the same day may lead to unsatisfactory eradication rates and more side-effects.

Yao W, Zhao X, Gong Y, Zhang M, Zhang L, Wu Q, et al. Impact of the combined timing of PD-1/PD-L1 inhibitors and chemotherapy on the outcomes in patients with refractory lung cancer. ESMO Open. 2021;6(2):100094.

> *(2) Due to the lack of a control group, we cannot assess the advantages and disadvantages of this treatment strategy compared to standardized neoadjuvant immuno-chemotherapy. We can refer to historical data. In the current clinical trials on*

> *neoadjuvant chemotherapy combined with immunotherapy (CheckMate-816, etc), what is the proportion of patients who had their chemotherapy reduced due to adverse reactions? Is there a difference in their efficacy? This could serve as a good historical reference.*

Thanks for your comment. In checkmate816, the rate of off neoadjuvant treatment in treatment group and control treatment group is 5.7% and 6.8% respectively. No patients have reduced their chemotherapy dosage due to intolerable side effects. However, it's a excellent suggestion to find a historical refence, so we will check details in other clinical trials.

> *(3) Among the 38 patients, there are 21 cases of SCC and 17 cases of LUAD. From the protocol, it can be seen that SCC patients had both albumin-bound paclitaxel and cisplatin reduced, whereas LUAD patients did not have a reduction in pemetrexed, only in cisplatin. Considering the different pathological subtypes and treatment strategies, I suggest the author to present the efficacy data for SCC and LUAD separately rather than combining them together.*

Thanks for your comment. In this cohort of 31 patients who underwent pathological evaluation, the ratio of squamous cell carcinoma (SCC) to lung adenocarcinoma (LUAD) was 16 vs 15. Upon comparing the groups, no statistically significant difference was observed in the treatment efficacy between SCC and LUAD patients.

Author response table 1.

Groups	SCC	LUAD	Chisq.test
pCR	7	5	p = 0.82
npCR	9	10	
MPR	12	6	p = 0.1
nMPR	4	9	

> *(4) In the discussion, the authors mention that during the adjuvant treatment phase, "no significant change was observed in evenness or clonality of TCR" (Page 13, Line 364). However, in Figure 3E, it can be seen that the evenness and clonality of TCR during the adjuvant treatment phase (i.e., C3) are significantly increased (P < 0.05).*

Thanks for your comment. For the TCR repertoire evenness and clonality, we present these metrics in Fig. S2 B-C. Throughout the treatment process of all patients, there were no significant changes in the Pielou index (representing evenness) or clonality. In Fig. 3E, we defined TCR clones with a frequency greater than 0.001 as "large clones" and examined their changes during cycle 1 and cycle 3. Therefore, although there was a significant increase in large clones during cycle 3, the overall TCR evenness and clonality did not show notable changes.

> *(5) The authors indicated that low-dose chemotherapy does not inhibit TCR expansion; however, due to the lack of a control group, we cannot conclude that "standard doses would affect TCR expansion." To better explore this possibility, please analyze the differences in TCR expansion between patients with bone marrow suppression and those without.*

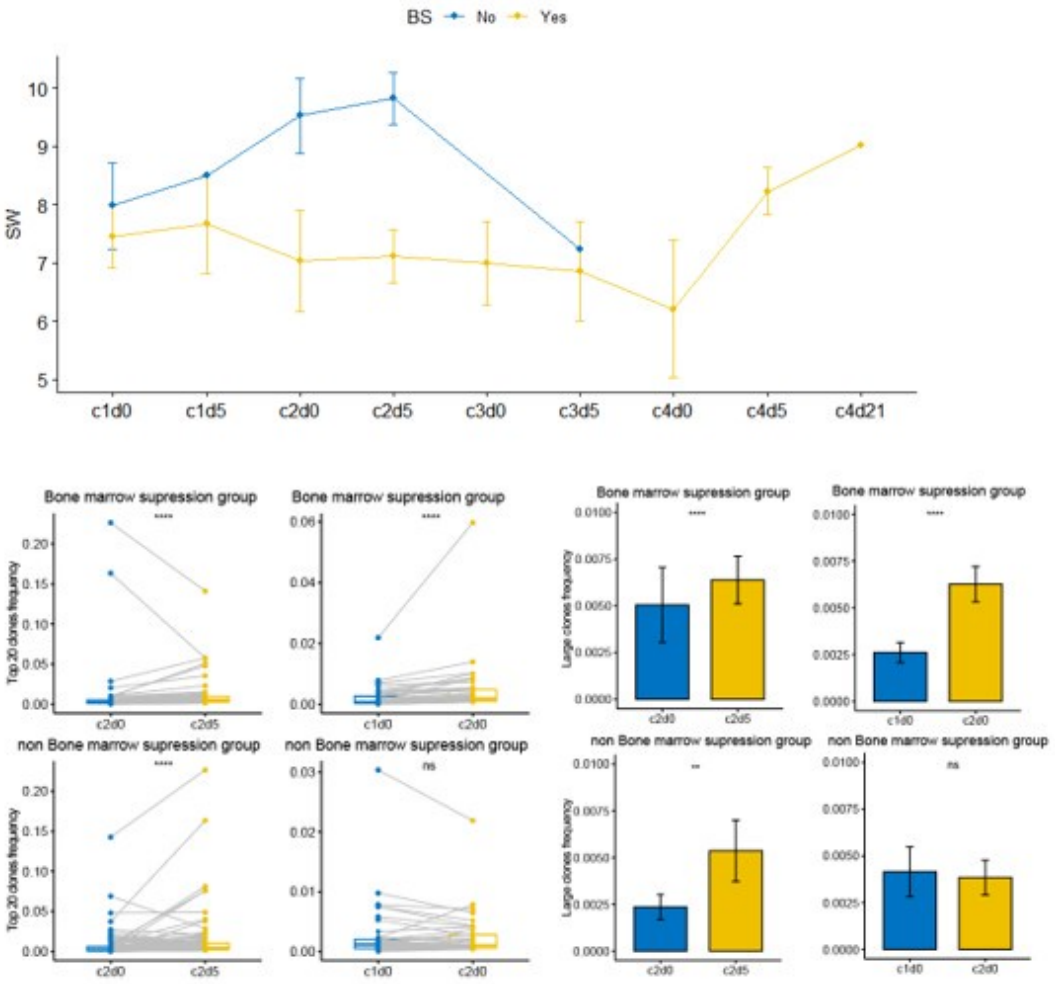
We analyzed the incidence of bone marrow suppression in patients who underwent blood TCR testing. The statistical results are shown in the figure below. Patients were grouped based on the presence or absence of bone marrow suppression to compare differences in TCR

clonal dynamics between the two groups during neoadjuvant therapy. As shown in the figure below, patients in the non-bone marrow suppression group exhibited higher TCR diversity (SW score) during treatment compared to those in the bone marrow suppression group. During neoadjuvant therapy, the dominant clones in both groups significantly increased from c2d0 to c2d5. However, from c1d0 to c2d0, there was no significant change observed in the non-bone marrow suppression group, possibly due to the limited sample size. Additionally, Patient P11 in the non-bone marrow suppression group showed a downward trend in dominant clones from c1d5 to c2d0, which may have influenced the overall results for this group during this phase.

Author response table 2.

Patients	WBC	HGb	PLT	Bone marrow suppression
P1	II	III	0	Y
P2	I	I	0	Y
P3	0	I	0	Y
P4	0	0	0	N
P5	I	0	0	Y
P6	I	0	0	Y
P7	0	2	0	Y
P8	0	0	0	N
P9	0	0	0	N
P10	III	0	0	Y
P11	0	0	0	N
P12	0	I	0	Y

Author response image 4.

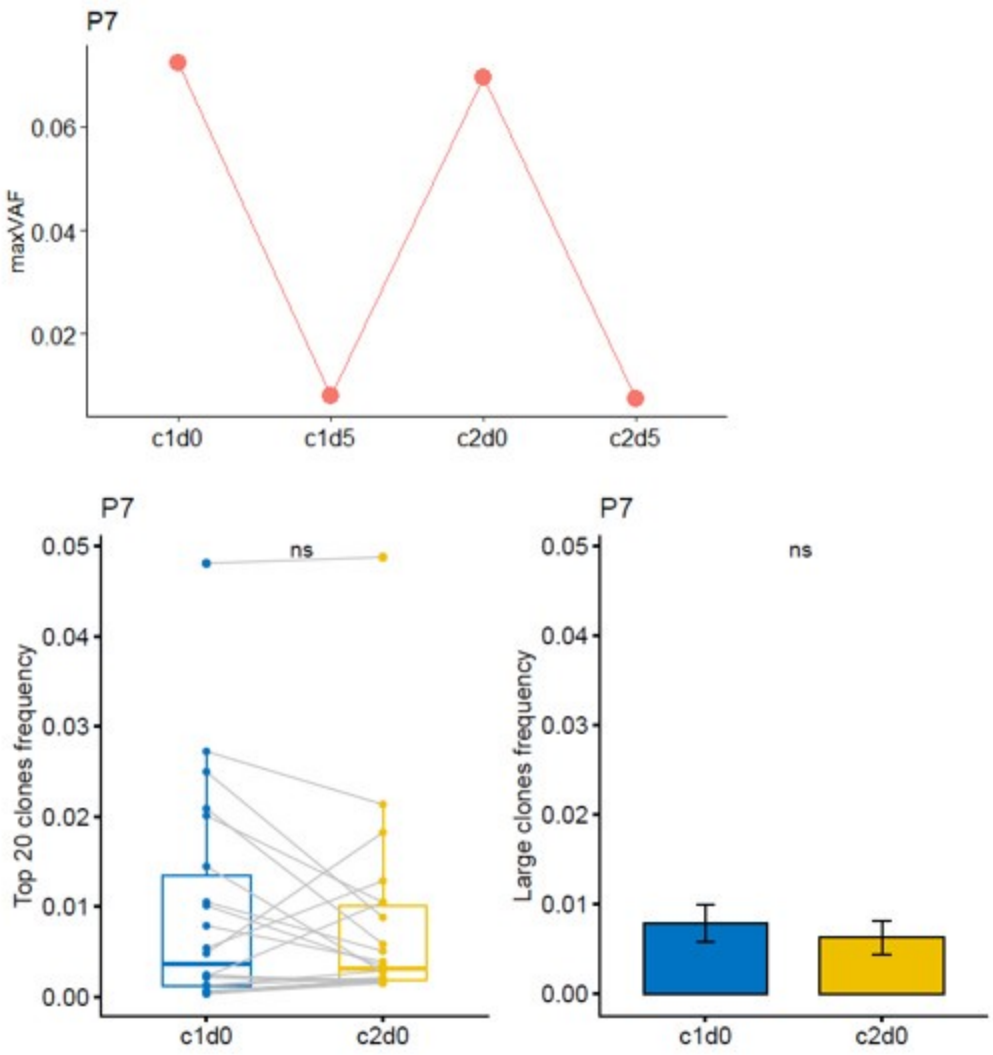


> *(6) In the analysis of ctDNA maxVAF, I noticed that one patient showed a significant drop at T1 (after C1 chemotherapy), followed by a notable rebound at T2 (after C1 delayed immunotherapy), and then a decline again at T3 (after C2 chemotherapy). Theoretically, maxVAF can reflect tumor burden and should change in accordance with treatment response. Could this indicate that the patient has a poor response to the delayed immunotherapy without concurrent chemotherapy? Additionally, please examine this patient's efficacy separately. What is the status of dynamic TCR? Does it show a trend opposite to that of maxVAF?*

Thanks for your comment. For Patient P7, the radiological evaluation reached PR, while the pathological assessment was non-MPR. The naming of time points has been revised according to the requirements: T0, T1, T2, and T3 were changed to c1d0, c1d5, c2d0, and c2d5, respectively. Combining both radiological and pathological evaluations, the patient experienced a certain degree of tumor shrinkage during neoadjuvant therapy but still retained some residual tumor cells. Theoretically, maxVAF can reflect the tumor burden in the bloodstream as a real-time indicator of treatment response. For patients with long-term benefits, maxVAF is expected to decrease as tumors are eliminated. However, in the short term, the release of large amounts of clonal ctDNA from destroyed tumor cells may lead to a temporary increase in maxVAF. Therefore, it is not possible to conclude that this patient had an adverse response to delayed immunotherapy based on individual cases. The increase in

maxVAF from c1d5 to c2d0 might result from the extensive release of newly exposed antigens. During this period, the top 20 and large clone TCRs did not show significant changes, suggesting that the patient's immune response was insufficient, leading to suboptimal neoadjuvant treatment efficacy and failure to achieve MPR. Additionally, there were no noticeable changes in maxVAF or TCR metrics from c1d0 to c2d0 for this patient, indicating that there is no evidence to suggest an inverse trend between TCR and maxVAF.

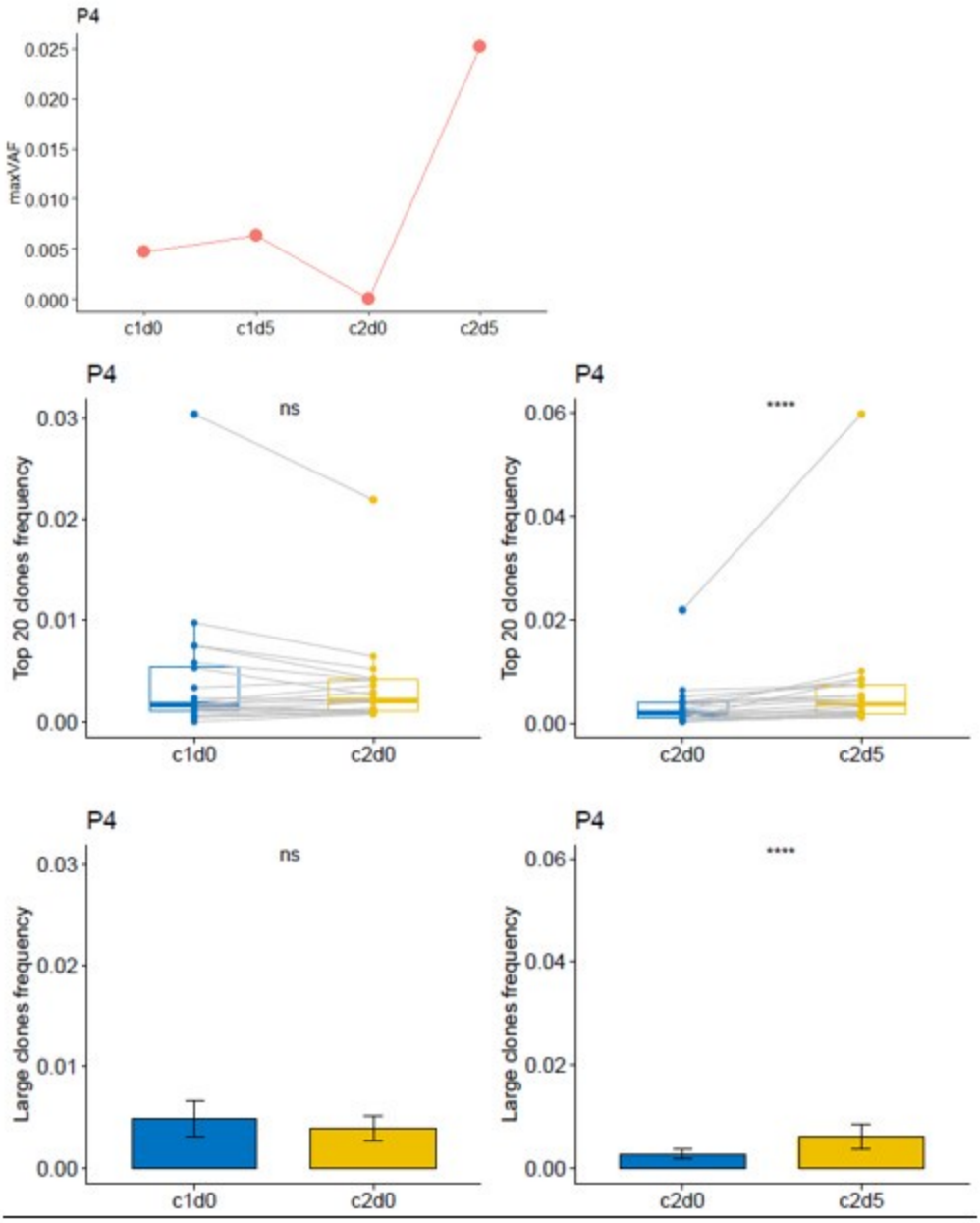
Author response image 5.



> *(7) In line with the previous question, another patient's maxVAF shows a significant rebound at T3. Please examine this patient's efficacy as well as the status of dynamic TCR.*

Thanks for your comment. For Patient P4, the radiographic assessment showed SD, while the pathological assessment indicated a MPR. Although the reduction rate of the tumor volume in this patient was low, the tumor cell content within the lesion was less than 10%, which suggests that this patient had a good response to neoadjuvant therapy. From c1d0 to c2d0, the maxVAF of this patient showed a downward trend, while there was no significant change in the dominant clone indices of the TCR. From c2d0 to c2d5, both the maxVAF and the TCR dominant clone indices increased significantly. This implies that this patient had a stronger immune response level compared to Patient P7.

Author response image 6.



> *Minor Comments:*
>
> *(1) Figure 2E shows only OS, but the corresponding description in the text mentions that OS and DFS are not reached.*

Thanks for your comment. Both OS and disease-free survival (DFS) records are available in Table S1. By January 31, 2025, the follow-up data were updated for 31 patients in Supplementary Table1. Among them, three patients experienced tumor recurrence, one of whom passed away. Additionally, seven patients were lost to follow-up. As a result, neither the overall survival (OS) nor the progression-free survival (PFS) reached the median number of events required for analysis. Since neither OS nor DFS have reached their median values, we opted to display only the OS in Fig. 2E.

> *(2) In the Discussion section, it is mentioned that there is controversy regarding chemotherapy combined with immunotherapy. I disagree with this statement. I believe that chemotherapy combined with immunotherapy is a consensus. The wording should be revised accordingly.*

Thanks for your comment. Yes, as you said, the combination of chemotherapy and immunotherapy has become a consensus. What we want to express is that how to optimize the administration time and dosage is worth further exploration. We will make a revise accordingly (Discussion line 328-331).

> *(3) The authors mentioned that the study involves multi-omics, but only ctDNA and TCR levels are included, with no RNA-related content observed. Perhaps a different term could be used.*

Thanks for your comment. In this study, we employed a multi-omics approach involving whole transcriptome, ctDNA, and TCR sequencing to investigation. RNA-related analyses are presented in Figure S3. Given that our primary focus is on the impact of this modified treatment on immune cells, we utilized RNA sequencing results to estimate immune cell compositions using the xCell and immunCellAI algorithms. The estimated immune cell profiles have been added to Supplementary Tables 5 and 6.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *Additional comment to the authors:*
>
> *The methods section refers to mRNA sequencing of the tumour tissue to define immune cell populations. Figure 3A also identifies that up to two timepoints were to be sequenced for individual patients. I could not find the results in the document.*
>
> *Please review the methods section and remove experimental methods where no data are presented.*

Thanks for your comment. As shown in Fig. 3A, for the mRNA data, we sampled and sequenced five patients (P1, P2, P4, P5, P7) before treatment. During the surgical phase, we sampled and sequenced three patients (P2, P5, P6). Then we utilized RNA sequencing results to estimate immune cell compositions using the xCell and immunCellAI algorithms. The estimated immune cell data have been added to Supplementary Tables 5 and 6. The T cells proportion comparisons were shown in fig. S3. The description of Whole transcriptome sequencing and immune cell abundance estimation were detailed in methods section.

https://doi.org/10.7554/eLife.99720.2.sa0