Jiao *et al. Molecular Cancer* (2025) 24:143
https://doi.org/10.1186/s12943-025-02332-8

Molecular Cancer

RESEARCH Open Access

Perioperative nivolumab and chemotherapy in locally advanced squamous cell carcinoma of the oesophagus: a randomized multicentre phase 2 study with circulating tumor DNA dynamics monitoring

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Abstract

Background Although neoadjuvant chemotherapy and immunotherapy show promise in treating oesophageal squamous cell carcinoma (OSCC), long-term survival data are limited. This randomized, multicenter phase 2 study evaluated the efficacy of perioperative Nivolumab with chemotherapy, followed by surgery and adjuvant immunotherapy, in patients with locally advanced resectable OSCC, and explored the prognostic role of circulating tumor DNA (ctDNA) status.

Methods In this trial, participants recruited from five centers were randomly assigned in a 2:1 ratio to receive either perioperative Nivolumab or a placebo in addition to chemotherapy (cisplatin and paclitaxel), followed by minimally invasive esophagectomy. For those who did not achieve a pathological complete response (pCR), adjuvant treatment with Nivolumab was administered. The main measure of success was the pCR rate, with secondary endpoints including the R0 resection rate, event-free survival, and overall survival. All outcomes and safety measures were assessed based on the intention-to-treat population. ctDNA levels were monitored as exploratory endpoints.

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Results Ninety patients were enrolled and randomized to Nivolumab or placebo plus chemotherapy. The pCR rate was slightly higher in the Nivolumab group (15%) compared to the control group (13.3%) (relative risk, 1.13; 95% CI, 0.38 to 3.36). No significant differences were observed in R0 resection rates (96.4% vs. 96.6%; $P > 0.05$). The median follow-up duration was 24.9 months (interquartile range: 22.8 to 26.7 months). Two-year event-free survival rates were 63.11% in the Nivolumab group versus 60.47% in the chemo group (hazard ratio, 0.97; 95% CI, 0.49 to 1.92). Two-year overall survival rates were 83.32% and 79.4%, respectively (hazard ratio, 0.82; 95% CI, 0.29 to 2.31). All participants were ctDNA positive at baseline, but post-treatment, 89% of the Nivolumab group and 62.5% of the placebo group turned ctDNA negative ($P = 0.01$). Those negative for ctDNA at all testing points showed significantly better disease-free survival ($P < 0.001$).

Conclusions Perioperative Nivolumab plus chemotherapy is a viable and safe option for systemically treating locally advanced resectable OSCC. Monitoring minimal residual disease through ctDNA could be potentially valuable for assessing the effectiveness of adjuvant therapy and for prognostic evaluation in a systemic manner.

Trial registration ClinicalTrials.gov registration NCT05213312.

Keywords Oesophageal squamous cell carcinoma (OSCC), Perioperative immunotherapy, Nivolumab, Chemotherapy

Introduction

Oesophageal cancer poses a significant health risk globally. East Asia records the highest incidence rates worldwide for both men and women, with oesophageal squamous cell carcinoma (OSCC) being the predominant histological subtype [1]. The standard treatment for locally advanced resectable OSCC is neoadjuvant chemoradiotherapy (nCRT) with surgery, improving survival over surgery alone [2, 3]. However, the JCOG1109 and our phase 3 CMISG-1701 trials showed no significant improvement in overall survival (OS) with nCRT over neoadjuvant chemotherapy (nCT), despite better local tumor control [4, 5]. This suggested that improving systemic treatment may improve long-term survival. In East Asia, nCT has been extensively adopted, showing significant results, particularly for patients with multiple lymph node metastases where radiotherapy is not feasible [6–8]. The advent of immunotherapy has transformed the treatment landscape for esophageal cancer. Immune checkpoint inhibitors (ICIs), such as programmed cell death protein-1 and its ligand (PD-1/PD-L1), have been effective in treating advanced or metastatic esophageal cancer [9–11]. Therefore, combining ICIs with nCT may improve systemic treatment survival outcomes.

A regimen combining nCT and nivolumab, an anti-PD-1 antibody, has improved pathological complete response (pCR) rates and extended OS in resectable non-small-cell lung cancer (NSCLC) [12, 13]. In esophageal cancer, however, the effectiveness of neoadjuvant immunotherapy (nIT) is still under investigation, awaiting more conclusive data. The initial pilot study combining nivolumab with nCT in 2019 enrolled sixteen patients with stage II/III esophageal/gastroesophageal junction (E/GEJ) cancer and achieved a pCR rate of 40% [14]. A phase 2 trial (NICE) updated its two-year follow-up data, showing two-year OS and PFS of 78.1% and 67.9%, respectively [15]. The ESCORT-NEO/NCCES01 trial, China's first phase 3 trial combining ICI with nCT, reported preliminary results with pCR rates of 28.0% and 15.4% in the ICI plus nCT groups, significantly higher than the 4.7% in the nCT-only group, meeting the primary pCR endpoint. However, data on event-free survival (EFS) remains immature [16]. Current trials lack long-term survival data, highlighting the need for robust evidence on the optimal ICI and nCT combination and its potential to enhance survival. Further, the Checkmate-577 trial highlighted ICIs' role in adjuvant therapy for non-pCR patients after nCRT [17], raising questions about their necessity in neoadjuvant therapy and tumor regression.

The ability to predict the effectiveness of neoadjuvant therapy and swiftly detect relapses could improve survival rates. Monitoring minimal residual disease (MRD) through circulating tumor DNA (ctDNA) has been linked to early recurrence and poorer prognosis across various cancer types [18–21]. Specifically in esophageal cancer, patients with detectable ctDNA post-chemoradiotherapy exhibited notably reduced PFS and worsened OS [22]. Furthermore, preliminary findings from a phase Ib trial on esophageal/gastroesophageal junction (E/GEJ) cancer indicated that patients with undetectable ctDNA levels before and after nIT experienced extended recurrence-free survival (RFS) and OS. These reports suggest that ctDNA clearance might reflect neoantigen-specific T-cell responses [23].

Building on the findings described above, we initiated a two-arm, multicenter, randomized, double-blind phase 2 study to compare the efficacy of systemic treatment combining nCT with immunotherapy against nCT alone for OSCC patients. We also conducted exploratory analyses to investigate the monitoring of ctDNA dynamics as a systemic biomarker for assessing pathological response and predicting survival outcomes.

Methods

Study design, eligibility criteria and participants

This phase 2, randomized, prospective, double-blind study enrolled 90 patients at five hospitals in China (ClinicalTrials.gov registration: NCT05213312, preregistered in December 2021). The primary protocol and all amendments received approval from the Institutional Review Board (IRB) of Zhongshan Hospital affiliated with Fudan University (B2022-004R) and from the IRBs of each participating institution. The study was performed following the Declaration of Helsinki and adhered to international standards of good clinical practice. Eligible participants were aged 18 to 75 years, diagnosed with stage II-III OSCC in the thoracic cavity based on the American Joint Commission on Cancer (AJCC) eighth edition staging system, and had histologically confirmed disease. Patients who had previously received anti PD-1/PD-L1 therapy or who had unqualified stages or histology were excluded.

The determination of sample size is described in the *supplementary appendix and* other details of the study protocol have been published previously [24]. The first and last participants were enrolled on May 15, 2022, and November 28, 2022, respectively.

Trial treatments

Based on prior completion of the treatment regimen by patients with OSCC and NSCLC in our center, as well as efficacy in other pre-trials, we ethically considered randomizing more patients to the Nivo + chemo group, which had better efficacy. Consequently, patients were randomly assigned in a 2:1 ratio to either the nivolumab plus chemotherapy group (arm A, Nivo + chemo group) or the placebo plus chemotherapy group (arm B, control group), followed by minimally invasive esophagectomy (MIE) (Figure S1).

In arm A (Nivo + chemo group), a fixed dose of 360 mg nivolumab (Bristol Myers Squibb) was administered intravenously on day 1, followed by cisplatin (80 mg/m^2, Pfizer) and paclitaxel (175 mg/m^2, Bristol Myers Squibb) on day 2. In arm B (control group), patients received cisplatin and paclitaxel at the same doses as that in the Nivo + chemo group. Following protocol, all enrolled patients were scheduled to receive two cycles of neoadjuvant therapy and then undergo MIE after a waiting period of four to six weeks. All MIE procedures were performed using the McKeown three-stage esophagectomy technique (cervical, thoracic, and abdominal incisions) with comprehensive two-field lymph node dissection covering the mediastinum and upper abdomen. Cervical lymph node dissection was additionally performed for tumors located in the upper one-third of the thoracic esophagus.

After MIE, adjuvant immunotherapy was applied for non-pCR patients. The regimen included a 240 mg dose of nivolumab biweekly for sixteen weeks, followed by 480 mg every four weeks until disease progression, unacceptable toxicity, or one-year post-operation (a total of sixteen doses).

Endpoints

The primary endpoint was the rate of pCR, defined as the absence of any viable tumor cells in both the primary tumor site and retrieved lymph nodes following neoadjuvant treatment. Secondary endpoints included the R0 resection rate, which indicates no vital tumor at the proximal, distal, or circumferential resection margins; EFS, the time from randomization to the occurrence of any event such as death, disease progression and addition of new treatments; disease-free survival (DFS), the time from surgery to the disease recurrence; and OS, the time from randomization until death from any cause. Additional secondary endpoints were the occurrence of treatment-emergent adverse events (AEs). The severity of adverse reactions, except those related to surgery, was assessed using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTC AE v5.0). Moreover, biomarkers including PD-L1 expression and MRD were evaluated in tumor microenvironment and peripheral blood samples to correlate with clinical outcomes.

CtDNA sample collection, processing and sequencing

Tumor samples for Whole Exome Sequencing (WES) were obtained through endoscopic biopsy of the primary esophageal tumor lesion before the initiation of neoadjuvant treatment. Only samples with a tumor cell content of ≥ 30% were included in the study. Genomic DNA (gDNA) was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissue specimens using the MagPure FFPE DNA/RNA LQ kit (Magen, Guangzhou, China). Peripheral blood genomic DNA was also extracted from EDTA-anticoagulated peripheral whole blood or buffy coat samples using the MagPure Universal DNA Kit (Magen, Guangzhou, China), following the manufacturer's instructions. DNA samples that passed the quality criteria were subjected to WES library preparation, exome capture, quantification, and sequencing on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA), using with 2 × 151 bp paired-end reads, targeting a mean coverage of 500x for tumor samples and 150x for the paired normal samples, as previously reported [25].

Peripheral blood samples (approximately 20 mL) were collected at each predefined time point using Cell-Free DNA BCT tubes (Streck, La Vista, NE, USA). Plasma-derived cell-free DNA (cfDNA) was extracted employing the QIAamp Circulating Nucleic Acid Kit (Qiagen).

Following quality assessment, the cfDNA samples were subjected to the PROPHET (Patient-specific pROgnostic and Potential tHErapeutic marker Tracking) assay for the detection of MRD. High-depth unique molecular identifier (UMI)-based sequencing was performed on Illumina's NovaSeq 6000 platform in San Diego, CA, US, with the sequencing protocol specifying 2 × 151 bp paired-end reads, aiming for a raw sequencing depth of 100,000x for personalized panels.

All sample collection procedures adhered to standard clinical protocols, and written informed consent was obtained prior to the sample collection.

Personalized ctdna assay for MRD detection

The analyses of WES read data were conducted using the Illumina DRAGEN Bio-IT Platform (Illumina, Inc., San Diego, CA, US). The DRAGEN Bcl Convert pipeline v3.7.4 was utilized to generate FastQ files from raw BCL data. Fastp v0.23.0 was employed to trim adapters, and reads shorter than 50 bp were removed. The clean reads were then aligned to the human reference genome (NCBI GRCh37; hg19), with PCR duplicates marked for subsequent filtering. For quality control of all samples captured by the Human Core Exome panel, tumor and paired normal alignments were assessed for various QC parameters using in-house software to evaluate capture efficiency, coverage uniformity, and library complexity. Then single nucleotide variants (SNVs) and insertions/deletions (INDELs) were identified. Variants were considered valid if supported by at least five reads and a variant allele frequency (VAF) of at least 3%. Variants occurring with a population frequency greater than 0.5% in tumors were discarded as potential single-nucleotide polymorphisms (SNPs). Germline mutations were filtered by comparing the tumor VAF fold change to the matched normal VAF, removing those with a fold change < 3 or both VAFs > 10%. Variant annotation was performed using ANNOVAR [26] and SnpEff v4.3 [27]. Following internal guidelines, up to 50 high-priority variants with a minimum VAF of 3% were selected, excluding those in repetitive areas, regions with > 75% GC content, or homologous sequences. An in-house biotinylated capture probe pool was subsequently designed for each personalized panel.

cfDNA was sequenced using these personalized panels, and sequencing data were processed through UMI adapter extraction, mapping, and filtering. The statistical significance of detected somatic mutations was evaluated using a Poisson distribution [25] considering mutations significant at a p-value below 0.05. The overall significance of the samples was determined through a chi-square test. A ctDNA MRD-positive status was defined as having at least two significant mutations with a sample-level p-value below 0.005. The ctDNA fraction in plasma was estimated by evaluating multiple loci using the maximum likelihood (ML) method, as previously described [25].

Statistical analyses

All statistical analyses were performed using R software (version 4.2.1). A two-sided P-value of less than 0.05 was deemed significant unless stated otherwise. For continuous variables, an independent t-test was used for normally distributed data, while the Wilcoxon rank-sum test or Kruskal-Wallis test was applied for non-normally distributed data. Categorical variables were analyzed with Fisher's exact test. Spearman's correlation was utilized to explore potential associations. Kaplan–Meier (KM) curves and the log-rank test were applied to assess two-year EFS, DFS and OS. Hazard ratios (HR) and 95% confidence intervals (CI) were derived from univariable Cox proportional-hazards regression. Multivariable Cox regression was conducted to investigate the impact of clinical variables on survival outcomes. The median follow-up time was calculated using the reverse KM method [28].

Results

Patient characteristics

A total of 91 patients were initially recruited, with one being excluded for not meeting the inclusion criteria. This left 90 patients who were randomized to receive neoadjuvant therapy: 60 in the Nivo + Chemo group and 30 in the control group. Of these, 89 patients (98.9%) completed the neoadjuvant treatment as scheduled. Five patients in the Nivo + Chemo group did not proceed to surgery (Fig. 1). Specific reasons included three patients opting out of surgery, one experiencing disease progression that rendered them ineligible for surgery, and one patient passing away due to unrelated causes. The patient's demographic and clinical characteristics are shown in Table 1.

Efficacy and surgical outcomes

The result of primary endpoint was negative. In the Nivo + Chemo group, nine patients (15%, 95% CI: 7.1 to 26.57%) achieved pCR, compared to four patients (13.3%, 95% CI: 3.76 to 30.72%) in the control group, with no significant difference ($P > 0.05$), nor in the subgroups analysis (Table S1 and Figure S2). The number of patients showing complete tumor regression at the primary site (ypT0) was slightly higher in the Nivo + Chemo group at 11 (18.33%, 95% CI: 9.52 to 30.44%) compared to 6 in the control group (20%, 95% CI: 7.71 to 38.57%). The major pathological response (MPR) rate was the same in both groups at 33.3% (Table 2).

The pathological outcomes for both groups are summarized in Table 3. The R0 resection rate was comparable

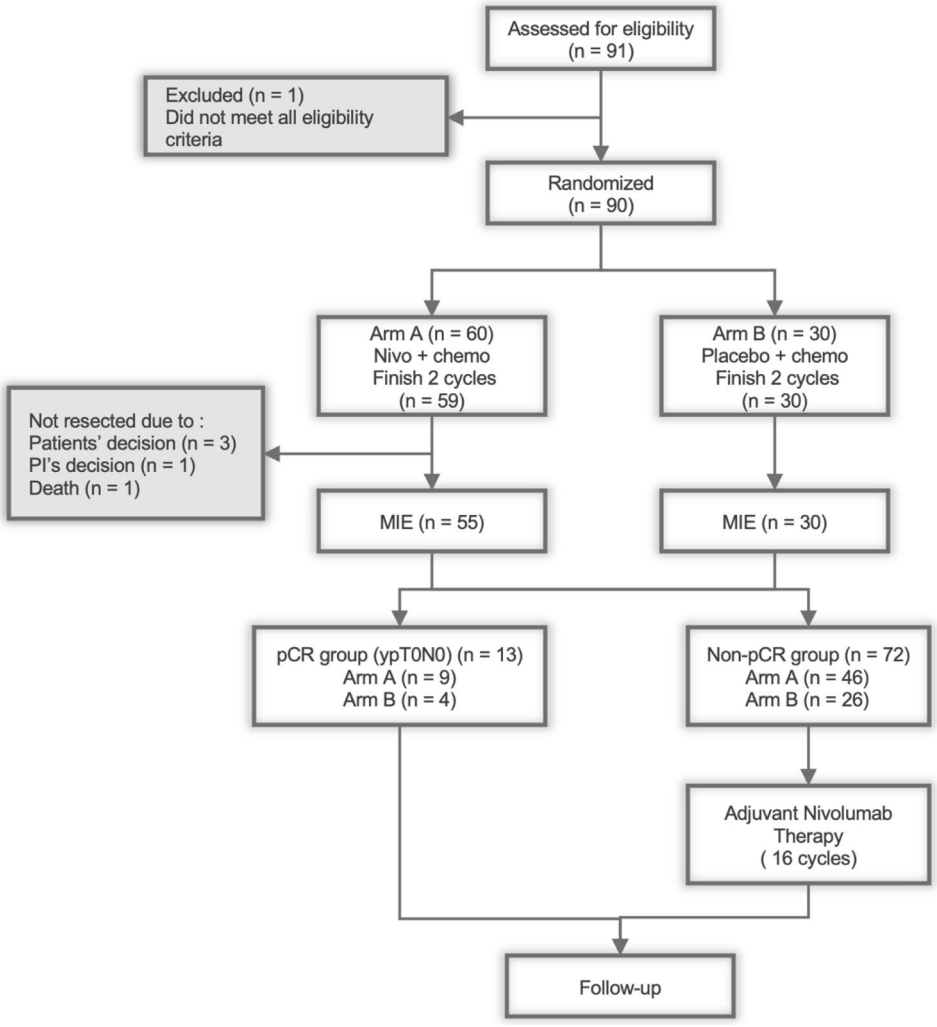

Fig. 1 Study Flowchart (CONSORT diagram)

in the two groups (Nivo + chemo vs. chemo, 96.4% vs. 96.6%, $P = 1$). Table S2 provides details on patients who did not achieve R0 resection and their subsequent treatments. Postoperative nodal staging revealed a significantly higher rate of negative retrieved lymph nodes in the Nivo + Chemo group compared to the control group (69.1% vs. 43.3%, $P = 0.021$).

In the control group, all patients proceeded to surgery, while in the Nivo + Chemo group, 55 patients (91.7%) underwent surgical procedures. The median time between the end of neoadjuvant therapy and surgery was 33 days, with an interquartile range of 31 to 35 days (Table S3). Surgical outcomes are detailed in Table 4. Intraoperative estimated blood loss was lower in the Nivo + Chemo group compared to the control group (74.62 ± 38.17 mL vs. 117.86 ± 88.42 mL, $P = 0.003$). There were no differences between the groups in operative duration, length of postoperative hospital stays, or number of lymph nodes retrieved (all $P > 0.05$). The postoperative ypTNM pathological staging is presented in Table S4. Postoperative complications occurred in 20 patients (36.4%) in the Nivo + Chemo group and 8 patients (26.7%) in the control group (Table S5). One patient from each group passed away during the perioperative period (within 30 days post-surgery) due to severe infections.

For patients who did not achieve a pCR, adjuvant monotherapy with nivolumab was implemented according to the study protocol. In the Nivo + Chemo group, 54.3% (25 out of 46) completed the adjuvant therapy, receiving an average of 11.7 cycles. In the control group, 57.7% (15 out of 26) completed their treatment, averaging 12.9 cycles. The reasons for discontinuing adjuvant therapy are detailed in Table S6. Patients who completed adjuvant therapy achieved better DFS than those who discontinued the treatment ($P < 0.0001$, HR, 10.62, 95% CI, 4.39 to 25.72, Figure S3A). However, there was no significant difference in DFS between those who completed adjuvant therapy and those who achieved pCR ($P > 0.05$,

Table 1 Baseline characteristics of ITT patients

Characteristic	Nivo + chemo (*n* = 60)	Chemo (*n* = 30)
Age (%)	62.59 ± 7.48	65.10 ± 6.42
< 65 years	33 (55)	16 (53.3)
≥ 65 years	27 (45)	14 (46.7)
Gender (%)		
Female	10 (16.7)	4 (13.3)
Male	50 (83.3)	26 (86.7)
Tumor location (%)		
Upper thoracic	9 (15)	4 (13.3)
Middle thoracic	34 (56.7)	16 (53.3)
Lower thoracic	17 (28.3)	10 (16.7)
BMI (%)	22.63 ± 2.87	23.72 ± 2.50
≤ 25	42 (70)	20 (66.7)
> 25	10 (16.7)	8 (26.7)
NA	8 (13.3)	2 (6.6)
ECOG (%)		
0	6 (10)	1 (3.3)
1	54 (90)	29 (96.7)
Median tumor diameter (mm)	14.84 ± 5.21	14.78 ± 6.35
Median tumor length (mm)	46.80 ± 18.76	45.44 ± 17.31
Clinical stage (%)		
cII	10 (16.7)	5 (16.7)
cIII	50 (83.3)	25 (83.3)
RECIST (%)		
PR	52 (86.7)	26 (86.7)
SD	4 (6.7)	4 (13.3)
PD	3 (5)	0
NA	1 (1.6)	0
PD-L1 expression – TPS (%)		
TPS < 1%	30 (50)	1 (3.3)
TPS 1–49%	15 (25)	2 (6.7)
Non-evaluable	15 (25)	27 (90)
PD-L1 expression – CPS (%)		
CPS < 1	12 (20)	0
CPS ≥ 1, < 5	20 (33.3)	0
CPS ≥ 5, < 10	7 (11.7)	2 (6.7)
CPS ≥ 10	6 (10)	1 (3.3)
Non-evaluable	15 (25)	27 (90)

Abv: BMI, body mass index; ECOG, Eastern Cooperative Oncology Group

HR, 2.02, 95% CI, 0.27 to 15.05). Among those who completed 16 cycles of adjuvant therapy in the control group, optimal DFS was observed ($P < 0.0001$, Log-rank for trend $P = 0.0412$). However, patients in the Nivo + Chemo group who did not complete adjuvant therapy had a lower median DFS compared to their counterparts in the control group (7.23 vs. 9.27 months, Figure S3B).

The median follow-up duration was 24.9 months (interquartile range: 22.8 to 26.7 months). As of the data cutoff, a total of 36 events were recorded, consisting of 23 events in the Nivo + Chemo group and 13 in the control group. There were 22 instances of disease recurrence (14 in the Nivo + Chemo group versus 8 in the control group)

Table 2 Pathological complete response

outcomes	Nivo + chemo (*n* = 60)	Chemo (*n* = 30)	*p*
pCR	9	4	0.832
pCR rate, % (95% CI) [a]	15 (7.1–26.57)	13.33 (3.76–30.72)	
Rate-diff, % (95% CI) [b]	1.67 (-16.08–15.37)		
RR (95% CI)	1.13 (0.38–3.36)		
ypT0	11	6	0.849
ypT0 rate, % (95%CI) [a]	18.33 (9.52–30.44)	20.00 (7.71–38.57)	
MPR	20	10	1
MPR rate, % (95%CI) [a]	33.33 (21.69–46.69)	33.3 (17.29–52.81)	

Abv: pCR, pathological complete response; CI, confidence interval; RR, relative risk; MPR, major pathological response

a. Clopper-Pearson method

b. Newcombe-Wilson method

and 14 deaths (8 in the Nivo + Chemo group versus 6 in the control group). Median EFS was reached in the control group (27.43 months), but there was no significant advantage observed in the Nivo + Chemo group compared to the control group in terms of EFS, DFS, or OS (Fig. 2). The overall two-year rates for EFS, DFS, and OS were 63.11%, 65.54%, and 83.32% in the Nivo + Chemo group, compared to 60.47%, 58.56%, and 79.4% in the control group, respectively.

Evaluation of personalized ctdna panels based on tumor tissue-based WES

A total of 324 blood samples were collected and sequenced from 65 patients to assess the prognostic potential of ctDNA-based MRD in patients with OSCC who underwent neoadjuvant therapy (Fig. 3A). The demographic and baseline clinical characteristics, such as age, sex, and TNM stage, were similar between the ctDNA-evaluable group and the intention-to-treat (ITT) population ($P > 0.05$, Table S7). The numbers for the blood samples collected at each time point including baseline (before neoadjuvant treatment), post-neoadjuvant therapy, post-surgical landmark point (within 2 months after surgery), and during follow-up were 64, 63, 43, and 154, respectively (Fig. 3A). The WES analysis of the biopsy specimens highlighted TP53 as the most commonly mutated gene in OSCC patients (Fig. 3B), which was consistent with the previous studies [29, 30]. To track ctDNA-based MRD, 2,533 variants were selected to design personalized panels, with the majority (2,139 or 84.4%) being patient-specific mutations (Fig. 3C).

Initially, all patients (100%) tested positive for ctDNA. The ctDNA levels were correlated with the sizes of the primary tumors at baseline ($R = 0.29$, $P = 0.029$, Fig. 3D). However, no significant differences in ctDNA levels were observed among patients with different clinical TNM stages, pCR status, or tumor regression grade (TRG) groups (all $P > 0.05$, Fig. 3E-G), indicating that the

Table 3 Pathological outcomes

	Nivo + chemo	Chemo	
outcomes	**(*n* = 55)**	**(*n* = 30)**	***p***
R0 resection (%)[a]	53 (96.4)	29 (96.6)	1
Tumor regression grade[a]			
1 (Residual tumor 0)	11 (20)	6 (20)	0.688
2 (Residual tumor 1–10%)	9 (16.4)	5 (16.7)	
3 (Residual tumor 11–50%)	12 (21.8)	4 (13.3)	
4 (Residual tumor > 50%)	23 (41.8)	15 (50)	
Lymph nodes involved[b]			
ypN0	38 (69.1)	13 (43.3)	**0.021***
ypN+	17 (30.9)	17 (56.7)	
ypTNM stage[b]			
I	27 (49.1)	11 (36.7)	0.088
II	11 (20)	2 (6.7)	
III	15 (27.3)	14 (46.6)	
IV	2 (3.6)	3 (10)	

a. Chi-square test

b. Fisher exact probability test

Table 4 Surgical outcomes

	Nivo + chemo	Chemo	
Outcomes	**(*n* = 55)**	**(*n* = 30)**	***p***
Surgical time, mean (SD), min	231.44 ± 49.85	227.61 ± 48.56	0.741
Estimated blood loss, mean (SD), mL	74.62 ± 38.17	117.86 ± 88.42	**0.003***
Postoperative hospital stays, mean (SD), d	10.71 ± 10.23	8.83 ± 2.73	0.335
Retrieved lymph nodes, mean (SD), No	28 ± 11.91	25.07 ± 10.08	0.256

baseline ctDNA status was not predictive of pathological response.

The association between preoperative ctDNA-based MRD and pathological response was then explored. All patients (11/11, 100%) who achieved a pCR had undetectable ctDNA levels, while 25% (13/52) of those who did not achieve pCR showed positive ctDNA, though this difference was not statistically significant ($P = 0.1$, Fig. 4A). Preoperative ctDNA status showed a strong association with different TRG groups ($P = 6.2e\text{-}4$, Fig. 4B). Figure S4A illustrates the changes in ctDNA levels from baseline to the preoperative period across pCR and TRG categories, highlighting the link between preoperative ctDNA status and pathological response. The ctDNA fraction at the preoperative timepoint significantly increased with higher TRG levels ($P = 0.016$, Figure S4B).

No difference in survival was observed between patients who achieved a pCR and those who did not, within both the ctDNA-evaluated and ITT populations (Fig. 4C and Figure S4C). However, patients with detectable preoperative ctDNA had significantly poorer DFS ($P < 0.001$; HR, 4.29, 95% CI, 1.68 to 10.93, Fig. 4D) and displayed a tendency towards worse OS ($P = 0.097$; HR, 3.31, 95% CI, 0.74 to 14.84, Figure S4D). Among patients who did not achieve pCR, those with positive preoperative ctDNA also had significantly worse DFS ($P = 0.0041$, Fig. 4E). A similar trend was also observed for OS ($P = 0.25$, Figure S4E). Multivariable Cox regression analysis confirmed that positive preoperative ctDNA status is an independent prognostic factor for poorer DFS, even after adjusting for traditional clinical variables ($P < 0.001$, Fig. 4F). These results implied that preoperative ctDNA status may be a more reliable prognostic biomarker than traditional tumor pathological response evaluations.

The relationship between preoperative ctDNA status and pathological outcomes was also investigated. Patients with detectable preoperative ctDNA predominantly presented with advanced ypT (III) and ypN (II/III) stages (Fig. 4G and H), which implied the presence of MRD for these patients. Neither pCR status nor TRG showed a significant association with treatment modality when comparing the Nivo + chemo group with the control group (Fig. 4I and Figure S4F). The negative rate of MRD was significantly higher in the Nivo + Chemo group compared to the control group (89% vs. 62.5%, $P = 0.02$, Fig. 4J).

Landmark ctdna status and longitudinal monitoring correlated with prognosis and adjuvant treatment efficacy

A total of 43 plasma samples were collected within two months post-R0 resection, serving as the landmark timepoint for analysis; 11 of these (25.6%) tested positive for

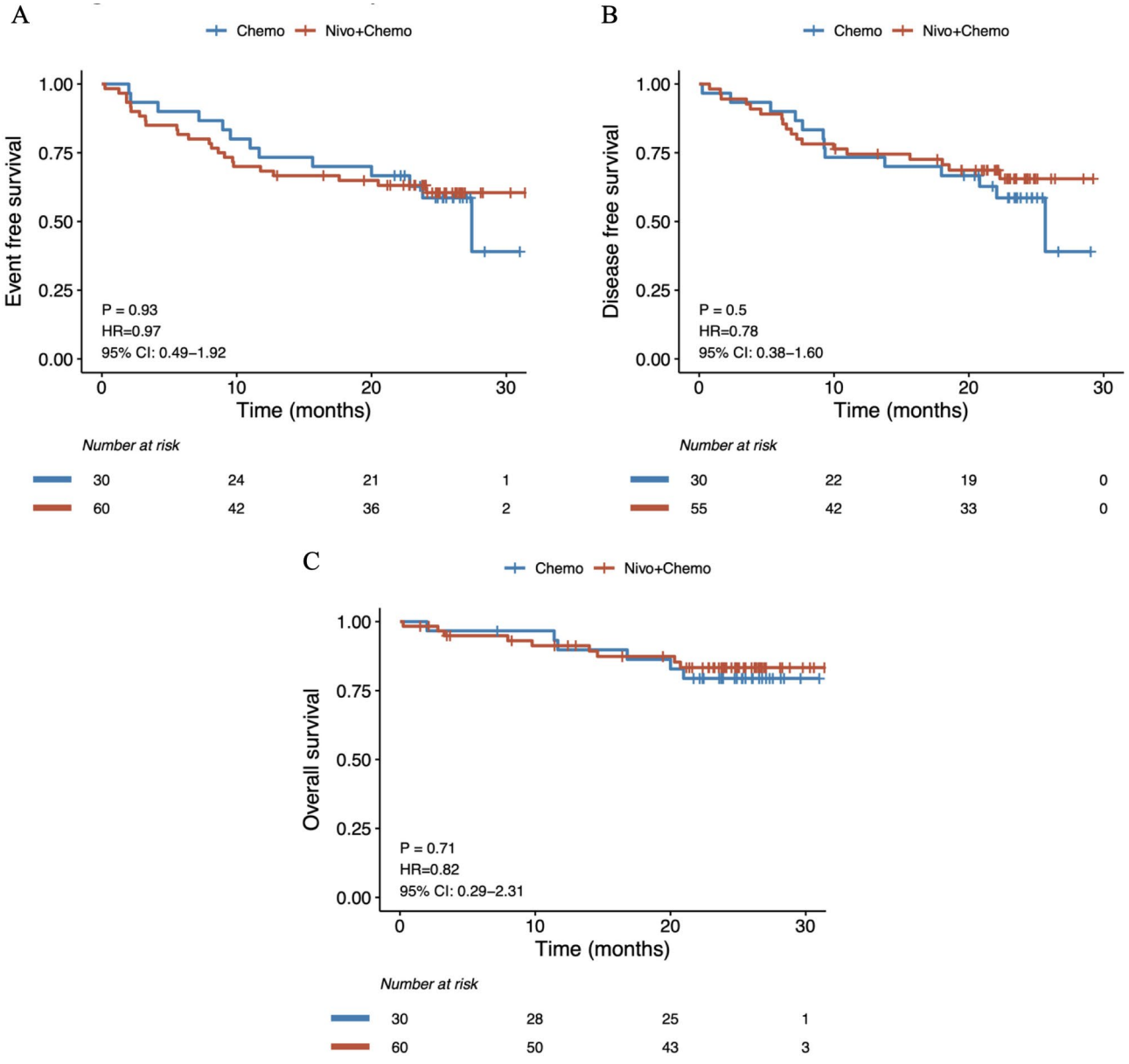

Fig. 2 Kaplan-Meier (K-M) curves of event-free survival (EFS), disease-free survival (DFS) and overall survival (OS). (**A**) K-M curves for EFS between patients in the Nivo + chemo and control group (intention-treat, ITT population). EFS at two-year was 63.11% and 60.47% in the Nivo + chemo group and the control group (hazard ratio[HR], 0.97, 95%CI, 0.49 to 1.92). The median EFS was 27.43 months in the control group and was not reached in the Nivo + chemo group. (**B**) K-M curves for DFS between patients underwent surgery in the Nivo + chemo and control group. DFS at two-year was 65.54% and 58.56% in the Nivo + chemo group and control group (HR, 0.78, 95%CI, 0.38 to 1.6). The median DFS was 25.7 months in the control group and was not reached in the Nivo + chemo group. (**C**) K-M curves for OS between patients in the Nivo + chemo and control group (ITT population). OS at two-year was 83.32% in Nivo + chemo group and 79.4% in control group (HR, 0.82, 95%CI, 0.29 to 2.31). The median OS were not reached in both groups

ctDNA. Patients with non-pCR and TRG4 had a higher incidence of positive landmark MRD and elevated ctDNA levels (Fig. 5A and Figure S5A&B). Landmark ctDNA patients exhibited significantly poorer DFS ($P < 0.001$; HR, 10.89, 95% CI, 3.22 to 36.83, Fig. 5B) and OS ($P < 0.001$, Figure S5C). Among non-pCR patients, those with positive landmark ctDNA had significantly worse DFS ($P < 0.001$, Fig. 5C) and OS ($P = 0.0032$, Figure S5D). The landmark ctDNA status was confirmed as an independent prognostic factor for DFS ($P < 0.001$) even after adjusting for clinical confounders (Fig. 5D). The landmark ctDNA status had a higher negative predictive value (0.88 vs. 0.8) and positive predictive value (0.73 vs. 0.62) than the preoperative ctDNA status in predicting relapse (Fig. 5E), indicating it as a precise DFS prognostic biomarker.

Patients with positive landmark MRD predominantly had ypTNM stages III/IV ($P < 0.001$, Fig. 5F), suggesting

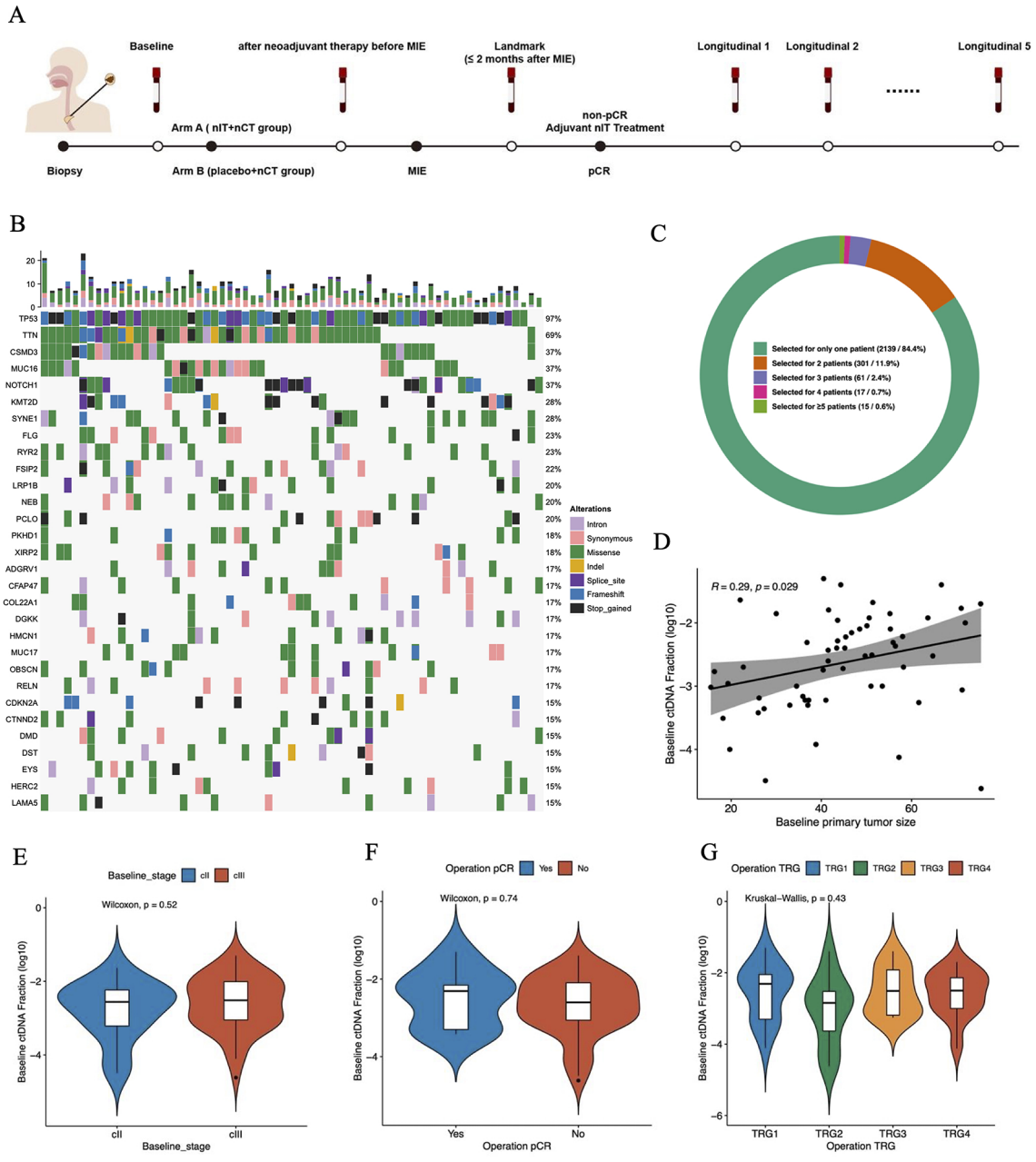

Fig. 3 Overview of the ctDNA analysis design and baseline ctDNA status. (**A**) Flow diagram of the ctDNA analysis design. (**B**) Genomic profile of the top 30 mutated genes for OSCC from the WES data. (**C**) Pie chart of the proportion of selected variants in personalized panels that were shared by patients. (**D**) Scatter plot between primary tumor size and baseline ctDNA fraction calculated by Spearman correlation ($R = 0.29$, $P = 0.029$). (**E**) Violin plot of the baseline ctDNA fraction for different pre-treatment clinical stage of tumor. ($P = 0.52$, Wilcoxon test). (**F**) Violin plot of the baseline ctDNA fraction for different operation pCR status. ($P = 0.74$, Wilcoxon test). (**G**) Violin plot of the baseline ctDNA fraction for different TRG stages. ($P = 0.43$, Kruskal-Wallis test)

a higher likelihood of residual MRD despite surgery. We then classified patients into three groups based on changes in ctDNA status from preoperative to landmark: consistently negative ($n = 31$), converted to positive ($n = 4$), and consistently positive ($n = 7$) (Figure S5F). As expected, patients with consistently negative ctDNA exhibited the best prognosis for both DFS ($P < 0.0001$, Fig. 5G) and OS ($P < 0.0001$, Figure S5E) than those with converted positive and consistent positive ctDNA status, most of which were of ypTNM III/IV stages ($P < 0.0001$, Fig. 5H). These findings underscore the significant prognostic value of landmark ctDNA assessment.

We expanded our personalized MRD panel to include post-surgery blood samples for ongoing monitoring. Patients with both landmark and follow-up samples were analyzed, yielding 149 longitudinal samples from 43

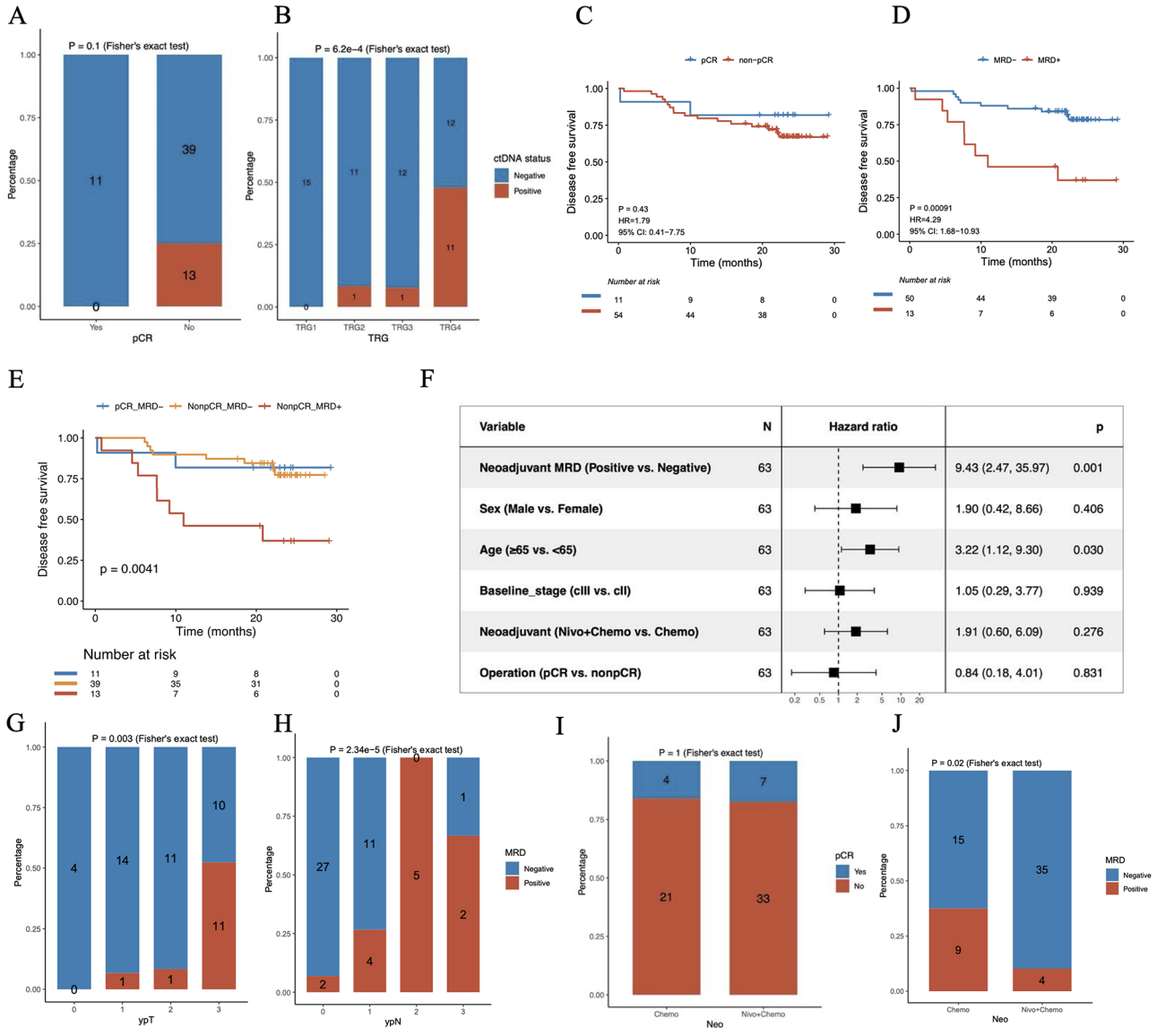

Fig. 4 Preoperative ctDNA status correlates with tumor regression and prognosis. (**A**) Comparison of preoperative ctDNA status in pCR and non-pCR. P values were calculated by Fisher's exact test. (**B**) Comparison of preoperative ctDNA status in TRG 1–4 group. P values were calculated by Fisher's exact test. (**C**-**D**) Kaplan-Meier (K-M) curves for disease-free survival (DFS) between distinct pCR (left) and preoperative ctDNA status (right). P values were calculated by log-rank test and hazard ratios (HR) were estimated by Cox proportional hazards model. (**E**) K-M curves for DFS analysis between patients stratified by pCR and preoperative ctDNA status. P values were calculated by log-rank test. (**F**) Coefficients of multivariable Cox model with clinical risk factors and preoperative ctDNA status. (**G**-**H**) Comparison of preoperative ctDNA status between different pathological tumor and nodal stages (ypT and ypN). P values were calculated by Fisher's exact test. (**I**-**J**) Proportion of patients for pCR and preoperative ctDNA status in different arms of neoadjuvant therapy (Nivo + chemo vs. Chemo). P values were calculated by Fisher's exact test

individuals. Any patient testing MRD positive at any time after surgery was classified as longitudinally MRD positive. A swimmer plot illustrates the treatment regimens, ctDNA statuses over time, and recurrence outcomes for each patient (Fig. 6A). Thirteen patients had longitudinally MRD positive, among them 10 relapsed. Conversely, of the 30 patients who consistently tested negative for longitudinal ctDNA, only 1 relapsed (Fig. 6A). Patients with positive longitudinal ctDNA status had worse DFS ($P < 0.0001$; HR, 19.65, 95% CI, 4.26 to 90.58) and OS ($P = 0.0032$) compared to those with a negative longitudinal ctDNA status (Fig. 6B). The longitudinal ctDNA status was independently associated with DFS after adjusting for other clinical variables (Fig. 6C).

At the landmark timepoint, no difference in DFS and OS was observed between patients with negative landmark ctDNA who received adjuvant therapy and those who did not (Fig. 6D). Since all patients with positive landmark ctDNA were non-pCR and received adjuvant therapy, comparing outcomes based on adjuvant therapy

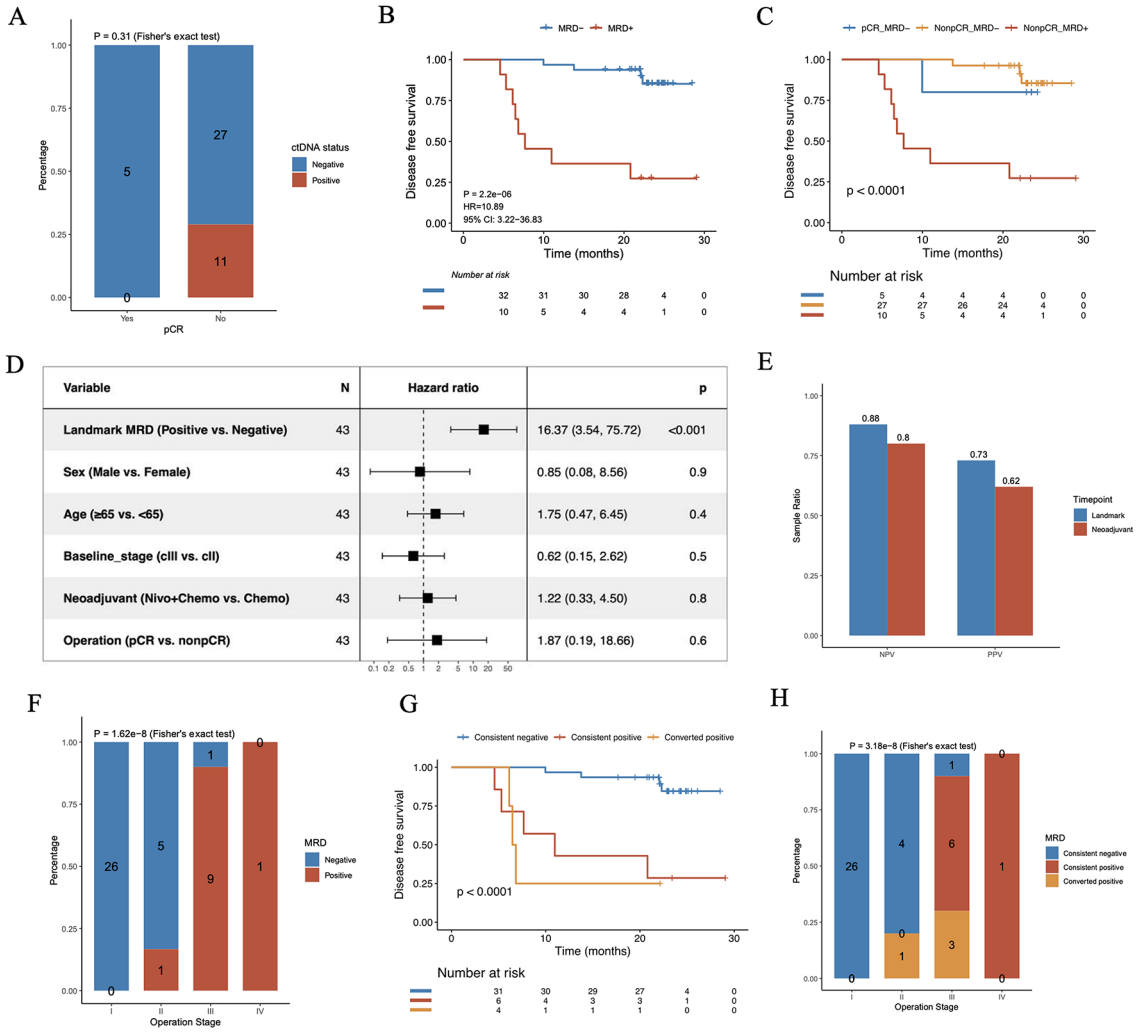

Fig. 5 Prognostic prediction by landmark MRD status. (**A**) Comparison of landmark ctDNA status between pCR and non-pCR patients. P values were calculated by Fisher's exact test. (**B**) Kaplan-Meier (**K-M**) curves between patients with different landmark ctDNA status for DFS. P values were calculated by log-rank test and HR were estimated by Cox proportional hazards model. (**C**) K-M curves for DFS analysis between patients stratified by pCR and landmark ctDNA status. P values were calculated by log-rank test. (**D**) Coefficients of multivariable Cox model with clinical risk factors and landmark ctDNA status. (**E**) Statistics of NPV and PPV for recurrence prediction by MRD at preoperative and landmark timepoints. (**F**) Comparison of landmark ctDNA status in different postoperative pathological stage of tumor. P values were calculated by Fisher's exact test. (**G**) K-M curves for DFS analysis between patients stratified by ctDNA status from preoperative to landmark timepoint including consistent negative, converted positive and consistent positive. P values were calculated by log-rank test. (**H**) Comparison of patients stratified by ctDNA status from preoperative to landmark timepoint in different postoperative pathological stage of tumor. P values were calculated by log-rank test

was not possible in this subgroup. Among the 34 patients who underwent adjuvant therapy and had both landmark and at least one subsequent MRD assessment, ten tested ctDNA positive at the landmark timepoint, and 24 tested negative (Fig. 6E). Among these ten patients, eight remained ctDNA-positive after adjuvant therapy, and seven of these experienced disease recurrence during follow-up. The remaining two patients, who turned ctDNA-negative following adjuvant therapy, did not experience any relapse. Among the 24 patients with negative landmark ctDNA, 22 remained consistently negative without relapse, while the two who turned positive both experienced recurrence (Fig. 6E). These results implied the significant potential of longitudinal ctDNA monitoring in assessing the effectiveness of adjuvant treatment.

Discussion

Neoadjuvant immunotherapy has proven effective across various cancers, and its role in OSCC is evolving from second-line to first-line treatment [31–33]. This trial evaluated perioperative nivolumab combined with chemotherapy, confirming feasibility and safety while significantly enhancing ctDNA clearance. This is the first study to assess this regimen in locally advanced OSCC while employing ctDNA-based MRD detection to monitor adjuvant therapy efficacy.

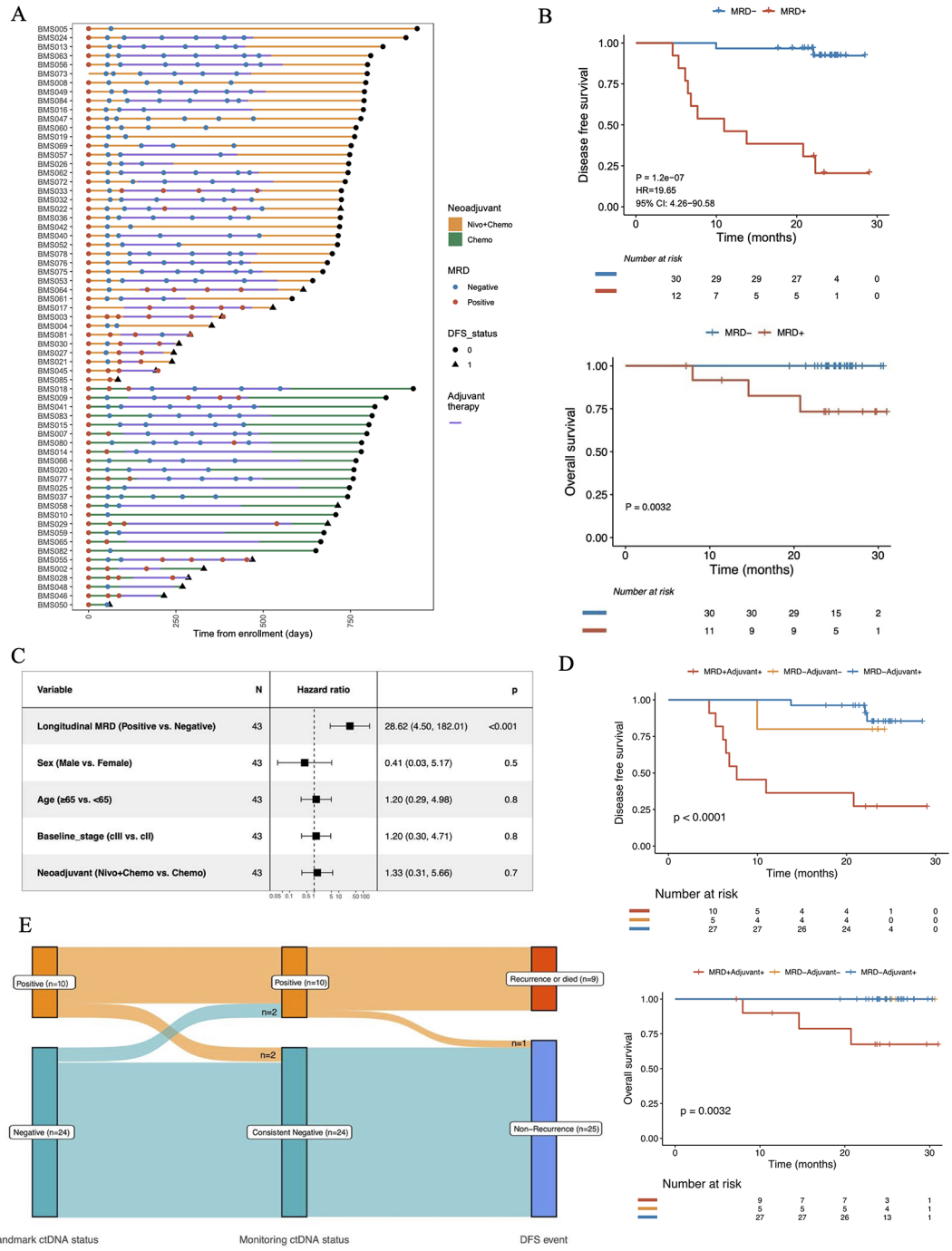

Fig. 6 Dynamic monitoring of longitudinal MRD. (**A**) Swimmer plot for 43 enrolled patients with distinct treatment options, ctDNA status at each time and recurrence status for longitudinal monitoring. (**B**) K-M curves between patients with different longitudinal ctDNA status for DFS and OS. P values were calculated by log-rank test and HR were estimated by Cox proportional hazards model. (**C**) Coefficients of multivariable Cox model with clinical risk factors and longitudinal ctDNA status. (**D**) K-M curves for DFS and OS between patients stratified by landmark ctDNA status and whether or not receiving adjuvant therapy. P values were calculated by log-rank test. (**E**) Sankey plot of ctDNA status at landmark and the follow-up monitoring timepoints, and the recurrence status for 34 patients receiving adjuvant therapy

The primary endpoint, the difference in pCR rates between the two groups, was not statistically significant (15% vs. 13.3%, $P = 0.832$). The pCR rate in our Nivo + chemo group aligned closely with that of the ICIs plus TP (paclitaxel plus cisplatin) group reported in the ESCORT-NEO trial. A limited two-cycle preoperative treatment, chosen for safety, may have contributed to the lower pCR rate compared with studies using three to

four cycles. Furthermore, the choice of the combination nCT regimen is critical; nab-TP, favored for its solubility and reduced risk of allergic reactions, has demonstrated higher pCR rates than TP in both the ESCORT-NEO study (28.0% vs. 15.4%) and other retrospective analyses (36.7% vs. 21.4%, $P = 0.018$) [16, 34].

The control group's pCR rate was 13.3%, higher than reported in other studies using the same TP regimen [4, 16, 35], likely due to the small sample size ($n = 30$) and the use of a higher cisplatin dose (80 mg/m^2) than is typical for Asian populations. Although increasing the nCT dosage may elevates pCR rates, it also increases the risk of treatment-related adverse events from cumulative toxicity, and the precise mechanism underlying the observed increase remains unclear.

While pCR rate serves as a valuable short-term indicator, its ability to predict long-term survival benefits is still uncertain [36–38]. Trials such as CMISG-1701 and JCOG1109 have shown that higher pCR rates following nCRT group do not necessarily correlate with improved OS [5]. In our study, no significant differences were observed in two-year OS, EFS, or DFS between the two groups, possibly because of similar pathological outcomes and the administration of adjuvant immunotherapy to patients who did not achieve pCR. Our results, consistent with the Checkmate577 trial, showed longer DFS in patients who completed adjuvant therapy (HR, 10.62; 95% CI, 4.39 to 25.72), with disease progression as the main reason for discontinuation (59.4%, 19/32) [17]. Additionally, we observed that patients in the control group who completed adjuvant therapy had better DFS outcomes compared to those in the Nivo + chemo group. This may be due to treatment resistance from earlier immunotherapy administration, following tumor immune cell depletion [39, 40].

Radical surgical resection remains the definitive method for confirming residual tumor, while both MIE and open resection are involved in significant anatomical alterations and reconstructions. Accurately predicting tumor regression preoperatively can guide treatment and help preserve cancer patients' quality of life [41, 42]. Liquid biopsy, through dynamic ctDNA monitoring, offers a promising solution [19, 23]. In our study, the PROPHET method which has previously demonstrated superior sensitivity and prognostic ability compared to fixed panel analyses, achieved a 100% baseline ctDNA-MRD positivity rate [25]. In the preoperative ctDNA assessments, we noted a higher clearance rate of ctDNA in the Nivo + chemo group (89% vs. 62.5%). However, only 22% (11/50) of patients with negative preoperative MRD successfully achieved pCR. Immunotherapy induces a specific tumor immune response, which may contribute to tumor cell eradication and suppression [43, 44]. This suggests that neoadjuvant therapy may eliminate hidden tumor foci in circulation, while primary tumor foci may not spread tumor cells. Interestingly, in a small cohort of OSCC patients treated with anti-PD-L1 or placebo combined with TP, all MRD-negative patients achieved a pCR (10/10), which was much higher than our proportion (100% vs. 22%) [45]. Thus, the ability of ctDNA to predict pCR merits further exploration.

Despite the accuracy of ctDNA analysis in detecting cancer recurrence, its cost is significantly higher than that of routine blood tests and radiographic evaluations. Optimizing the timing of ctDNA testing could reduce unnecessary sampling and thereby lessen both the physical and financial burdens on patients. Our monitoring of ctDNA-based MRD at multiple treatment stages showed that preoperative, landmark, and longitudinal ctDNA statuses were associated with recurrence, with MRD-negative patients enjoying longer DFS. These observations are consistent with previous studies in curative or locally advanced OSCC, where early ctDNA clearance was linked to improved progression-free survival and reduced recurrence risk [46, 47].

In addition to correlating with survival, ctDNA effectively predicts recurrence. In the preSINO trial, ctDNA-positive cases had a higher rate of postoperative distant recurrence than negative patients (15.1% vs. 3.3%) [48]. Similarly, in stage 2–3 gastric cancer, patients with positive ctDNA after adjuvant chemotherapy faced a markedly increased recurrence risk (HR = 14.99, 95% CI = 3.08–72.96) [49]. Moreover, during adjuvant nivolumab therapy, continuous ctDNA monitoring revealed an increasing HR (9.43, 16.37, 28.62) over time for patients with positive MRD status. Notably, MRD-negative patients receiving adjuvant therapy achieved superior DFS and OS, suggesting that sustained ctDNA clearance may be a beneficial effect of such treatment.

Collectively, these findings support the potential of MRD as a surrogate endpoint for predicting long-term survival outcomes, although assay sensitivity is crucial. Efforts to enhance sensitivity have focused on in vitro improvements, such as optimizing sample volume, library preparation, and bioinformatics analysis [50]. Martin-Alonso et al. developed two intravenous priming agents that temporarily slow cfDNA clearance in vivo, enhancing ctDNA concentrations and improving detection sensitivity for small tumors in mice by over 60% [51]. Nevertheless, challenges remain in standardizing ctDNA testing across platforms, managing high costs, and addressing ethical considerations.

This study has several limitations. First, because the primary endpoint was not met, additional follow-up is required to determine whether immunotherapy confers long-term survival benefits. Second, the COVID-19 pandemic impeded the collection of valid baseline tissue samples for some patients, limiting their inclusion

in MRD assessments. Finally, the relationships among ctDNA-MRD, pCR rate, and prognosis require further analysis and validation in larger phase 3 studies, and the underlying molecular mechanisms linking immunotherapy to ctDNA clearance warrant more in-depth investigation.

In conclusion, combining perioperative nivolumab with chemotherapy presents a viable and safe systemic treatment option for OSCC. MRD detection using ctDNA offers potential insights into the efficacy of adjuvant therapy and prognosis through systemic monitoring. This method could be instrumental in predicting early recurrence and facilitating the initiation of timely interventions. Compared with nCT alone, immunotherapy combined with nCT did not improve the pCR rate but demonstrated a superior effect on ctDNA clearance, suggesting the ability of ctDNA serving as a biomarker for preoperational evaluation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12943-025-02332-8.

Supplementary Material 1

Acknowledgements
We would like to express gratitude to the participating patients and their family. We also want to thank all the participating centers in the trial.

Author contributions
HJ, SL, JG, DJ and PC contributed equally to this trial. LT, JY and TL conceptualized and designed the research. LT was the principal investigator. HJ, JG, ZH, YF, HW, ML, HT, TJ, GL, SZ, HY, YY, ZL, BL, JX, XL, YH, LA, TL, JY and LT contributed to patient enrolment and data acquisition. SL, DJ, PC, FL, JW, XF did the statistical analysis. DJ and YH did the pathological analysis. SL, PC, XF and FQ did the visualization. GW, ZZ, SC did the medical reviewing. SL, PC, ZH, CL and LT drafted the original manuscript. All authors contributed to manuscript revision.

Funding
The study was funded by Bristol-Myers Squibb (No. CA209-6KP), whose role was limited to provision of financial support and nivolumab for patients. The article has been subjected to peer-review by the funding body.

Data availability
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The primary protocol and all amendments received approval from the Institutional Review Board (IRB) of Zhongshan Hospital affiliated with Fudan University (B2022-004R) and from the IRBs of each participating institution. The study was performed following the Declaration of Helsinki and adhered to international standards of good clinical practice.

Consent for publication
All sample collection procedures adhered to standard clinical protocols, and written informed consent was obtained prior to the sample collection.

Competing interests
Lijie Tan reported that the study was funded by Bristol-Myers Squibb (CA209-6KP). The other authors declared no competing interest.

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Received: 7 February 2025 / Accepted: 12 April 2025
Published online: 15 May 2025

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