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Anlotinib combined with benmelstobart as a chemo-free first-line treatment in advanced esophageal squamous cell carcinoma: an exploratory multicenter, single-arm phase II clinical trial

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Abstract

Background No combined antiangiogenic and PD-1/PD-L1 blockade therapy has been investigated as a chemo-free first-line treatment for advanced esophageal squamous cell carcinoma (ESCC). This study evaluates the efficacy and safety of anlotinib combined with benmelstobart as a chemo-free treatment in previously untreated advanced ESCC, and identifies potential predictive biomarkers using next-generation sequencing (NGS).

Methods ALTER-E-003, a single-arm, open-label phase II trial, enrolled patients with advanced ESCC across five Chinese centers. Patients received oral anlotinib 12 mg daily on days 1–14 per three-week cycle, with benmelstobart 1200 mg infused on day 1 of each cycle for up to 24 months. Thereafter, patients received anlotinib maintenance therapy. Primary endpoint was objective response rate (ORR). Secondary endpoints included progression-free survival (PFS), overall survival (OS), disease control rate (DCR), duration of response (DOR), and safety. NGS and fluorescent multiplex immunohistochemistry (mIHC) were performed on tumor specimens.

Results Of 53 screened patients, 46 completed the study. The confirmed ORR was 56.5% (95% CI 41.1–71.1), and DCR was 91.3% (95% CI 79.2–97.6). Median PFS was 15.74 months (95% CI 9.03–21.91). Treatment-related adverse events occurred in 93.5% of patients, with 28.3% experiencing grade 3 or higher events. NGS revealed a novel predictive mutational signature (*TP53+/FAT1+/NOTCH3-*) that was associated with better ORR (65.6% versus 11.1%, $P<0.001$), longer median PFS (17.91 versus 5.32 months, $P=0.005$) and improved OS ($P=0.006$).

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Conclusion First-line anlotinib-benmelstobart combination demonstrated durable responses and acceptable safety in ESCC patients. Exploratory biomarker analyses identified a *TP53+/FAT1+/NOTCH3-* mutational signature potentially associated with improved outcomes, though further validation in randomized trials is warranted.

Trial registration NCT05038813.

Keywords Anlotinib, Benmelstobart, Esophageal squamous cell carcinoma, Next-generation sequencing, TP53+/FAT1+/NOTCH3-, PD-L1

Background

Esophageal cancer was the 11th most common cancer worldwide in 2022, with 510,700 new cases, and ranked 7th in mortality, resulting in 445,100 deaths [1]. The overall 5-year survival rate for this disease is just 20% [2]. Approximately half of all esophageal cancer cases are found in Asia, particularly in China, where esophageal squamous cell carcinoma (ESCC) is the most prevalent subtype [3]. Many patients with ESCC are diagnosed at an advanced or metastatic stage, and despite undergoing the best available curative treatments, most ultimately experience a relapse.

Frontline systemic doublet chemotherapy, comprising 5-fluorouracil (5-FU) plus platinum in Western countries and Japan, or taxane plus platinum in China, is the standard treatment for advanced ESCC. This approach achieves a response rate of 30–40%, with progression-free survival (PFS) between 4 and 6 months, and overall survival (OS) ranging from 7 to 13 months [4–8]. Immune checkpoint inhibitors (ICIs) have the potential to transform the treatment landscape for ESCC. Trials such as KEYNOTE-590, CheckMate-648, ESCORT-1, and JUPITER-06 have shown that immunochemotherapy offers significant survival benefits over traditional doublet chemotherapy for advanced ESCC in the first-line setting [5, 9–13]. However, most of these trials included concurrent chemotherapy, which can lead to considerable renal and hepatic toxicities, negatively affecting patients' quality of life [6]. Patients with advanced ESCC often have compromised physical performance and may struggle to tolerate chemotherapy-related side effects. Additionally, ICI therapy may not always enhance the effects of chemotherapy and might not be suitable for every patient [14]. In CheckMate-648 trial after 29 months of minimum follow-up, nivolumab plus ipilimumab continued to show improved OS vs. chemotherapy alone in patients with tumor-cell programmed death ligand 1 (PD-L1) expression ≥ 1%, but without further PFS benefit. Importantly, the incidence of grade 3 or 4 treatment-related adverse events (TRAEs) was lower for nivolumab plus ipilimumab compared to chemotherapy alone (32% versus 36%) [15].

Antiangiogenic therapy could shift the immune suppressive tumor microenvironment towards a more immune active state [16]. However, apatinib combind with camrelizumab and doublet chemotherapy (liposomal paclitaxel and nedaplatin) for advanced ESCC in the first line setting was associated with a high rate of grade 3 or higher TRAEs (90%) [11]. Meanwhile, apatinib plus camrelizumab as second line treatment of advanced ESCC led to an objective response rate (ORR) of 34.6% and an incidence of grade 3 or higher TRAEs of 44% [17]. These findings prompted us to hypothesize that a dual approach targeting immune checkpoints and tumor angiogenesis could be both effective and safe as a frontline chemotherapy-free regimen for advanced ESCC, thereby minimizing chemotherapy-related toxicity. Benmelstobart (TQB2450), a humanized IgG1 anti-PD-L1 antibody, inhibits PD-L1's interaction with PD-1 and binds to CD80, showcasing antitumor activity. Anlotinib, a multikinase inhibitor, simultaneously targets vascular endothelial growth factor receptor (VEGFR) 1, 2 and 3, c-KIT, and PDGFR β and exerts broad inhibitory effects on oncoangiogenesis and is active against a variety of tumor types in clinical trials [18]. Anlotinib has also demonstrated prominent antitumor activities for recurrent or metastatic ESCC in second or later line setting. Together, anlotinib plus benmelstobart has shown good tolerabilities and promising antitumor activities in several cancer types as a second-line or later treatment [19–22]. Currently, there are limited chemotherapy-free options approved for first-line treatment of advanced ESCC.

Molecularly stratified therapies for ESCC have been hampered due to the lack of robust biomarkers that could guide combination immunotherapy. While extensive genomic and molecular characterization of ESCC has been conducted, PD-L1 remains the most established biomarker, with other potential biomarkers still requiring further validation [23–26]. In this study, we evaluated the efficacy and safety of combining anlotinib, an antiangiogenic agent, with benmelstobart, an anti-PD-L1 antibody, as a first-line treatment for advanced ESCC. Given the growing importance of biomarker-guided targeted therapies and the current lack of established biomarkers for concurrent immunotherapy and antiangiogenic therapy, we also conducted an exploratory analysis to identify potential predictive biomarkers for this combination treatment.

Methods

Study design and patients

ALTER-E-003 was a single-arm, open-label trial conducted at five centers across China. Adult patients between 18 and 75 years of age who had histologically or cytologically-confirmed, locally advanced, recurrent or metastatic ESCC that was not amendable to curative esophagectomy were eligible. Patients should have received no prior systemic therapy, or have relapsed more than 6 months after the end of prior (neo) adjuvant/radical treatment regimens (including radical surgical resection and radical radiotherapy regimens). Other key eligibility criteria are described in detail in Supplemental data I.

All patients provided written informed consent before any trial-related activities. The trial protocol (in Supplemental data II) was approved by the institutional review board or ethics committees of each participating site and undertaken in adherence with the principles of the Declaration of Helsinki and Good Clinical Practice Guidelines. The trial is registered with ClinicalTrials.gov (NCT05038813).

Study interventions

Patients received oral anlotinib (Chia-Tai Tianqing Pharmaceutical Co., Ltd.) 12 mg once daily on day 1 to 14 per cycle, with each cycle lasting for three weeks. Benmelstobart 1200 mg was infused on day 1 of each cycle for maximally 24 months. Thereafter, patients received anlotinib maintenance therapy. Treatment was continued until disease progression, death or unacceptable toxicities. Two dose reductions (12 mg/day to 10 mg/day and 10 mg/day to 8 mg/day) were allowed for anlotinib. The protocol-defined criteria for dose modification of anlotinib are detailed in Supplemental data I. Treatment interruption of benmelstobart was allowed for maximally three weeks. No dose modification was allowed for benmelstobart. Either drug was continued at the discretion of the investigators upon discontinuation of the other drug due to toxicities.

Assessments and endpoints

Disease assessment was made by investigators per RECIST version 1.1 at baseline, every two cycles for the first six cycles, and every three cycles thereafter. Complete response (CR) and partial response (PR) had to be confirmed radiologically at least four weeks and stable disease (SD) at least 6 weeks after an initial documentation. Progressive disease (PD) per RECIST version 1.1 was confirmed by iRECIST [27]. Safety monitoring included assessment of adverse events (AEs) and serious AEs using the Common Toxicity Standards of the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTC AE) version 5.0 throughout the study and until 30 days after the final dose of study drugs.

The primary efficacy endpoint was ORR, defined as the proportion of patients who achieved CR and PR as the best overall response per RECIST version 1.1. Secondary efficacy endpoints included progression-free survival (PFS), calculated from the date of medication initiation to the date of the first documented PD or death, whichever occurred first; overall survival (OS), calculated from the date of medication initiation to death from any cause; disease control rate (DCR), defined the proportion of patients who achieved CR, PR and SD as the best overall response; duration of response (DOR), calculated from the first documented CR or PR to the first documented PD or death, whichever occurred first.

Whole exome sequencing

Genomic DNA from formalin-fixed and paraffin-embedded (FFPE) sections and white blood cell control samples were extracted with the QIAamp DNA FFPE Tissue Kit and DNeasy Blood and Tissue Kit (Qiagen), respectively. Whole exome sequencing (WES) was performed to the target-enriched library on the HiSeq4000 NGS platform (Illumina) according to the manufacturer's instructions. Sequencing data were analyzed as previously described [28]. Tumor mutation burden (TMB) was calculated as the number of non-synonymous mutations per million bases in the coding region. The APOBEC-TMB was calculated based on the number of APOBEC signature-related mutations summarized using the R package 'Sigminer'. As for copy number analysis, the tumor purities of our data were first estimated using ABSOLUTE [29]. Purity-adjusted data were then used to calculate gene-level copy number variations (CNVs) using FACETS [30]. More details can be seen in the Supplemental data I.

Fluorescent multiplex immunohistochemistry

FFPE tissues were sectioned and deparaffinized. After antigen retrieval, multiplex immunohistochemistry (mIHC) was performed by AlphaXPainter® X30 (Alpha X Bio, Beijing, China) using two panels of primary antibodies against the proteins (Supplemental date I). Images were scanned with ZEISS AXIOSCAN 7 (ZEISS, Oberkochen, Germany) and analyzed with HALO software (v3.6; Indica Labs, USA). The cell positivity rate (%) was calculated as the number of cells positive for a specific marker within a given area divided by the total number of cells in the relevant area.

Gene set enrichment analysis (GSEA) and immune infiltrate analysis

An ESCC cohort ($n = 95$) was obtained from The Cancer Genome Atlas (TCGA) Firehose Legacy. RNA expression matrix and gene mutation data were acquired from

the cBioPortal database (https://www.cbioportal.org/study/summary?id=esca_tcga). Differential expression analysis was performed using the R package DESeq2 based on negative binomial distribution. GSEA was performed using the R package clusterProfiler (v4.2.2). The gene sets "h.all.v2023.2.Hs.symbols.gmt" were selected as reference. The enrichment of a pathway with P.adjust ≤ 0.05 and|normalized enrichment score| > 1 was defined as statistically significant. Immune infiltrating cells were assessed and quantified using MCP-counter [31].

PD-L1 expression analysis

PD-L1 staining was performed on formalin-fixed, paraffin-embedded tumor samples using PD-L1 clone 22C3. Combined positive score (CPS) and tumor proportion score (TPS) were calculated. CPS considered both tumor and immune cells, while TPS focused on tumor cells only. PD-L1 positivity was defined as CPS ≥ 1 or TPS ≥ 1% (Supplemental data I).

Statistical analysis

In this study, Simon's two-stage design was used to estimate the sample size [32]. The unilateral alpha value was taken as 0.05 and the beta value as 0.2, with a study certainty of 80%. The ORR of the first-line standard regimen of cisplatin combined with 5-FU regimen in advanced ESCC reported in previous studies ranged from 29.3–33.3% [9, 33]. It was hypothesized that the anlotinib hydrochloride combined with benmelstobart regimen could increase the ORR to 50% in such patients. Target accrual was a minimum of 15 patients in stage (1) If more than 5 of the patients achieved CR or PR, a minimum of 31 patients were required in stage (2) The study was considered to have met the primary efficacy end point when more than 18 patients in the two stages achieved CR or PR with anlotinib plus benmelstobart.

The study followed the intention-to-treat (ITT) principle. The full analysis set (FAS) included all patients who had received at least one dose of the study medications. Efficacy analysis was based on the ITT population. Fisher's exact test was employed to assess disparities in categorical variables across groups, while the Wilcoxon rank sum test was utilized to ascertain distinctions in continuous variables between these groups. For association analysis between mIHC result and clinical efficacy, the OS or PFS in the samples were sorted by time from smallest to largest, respectively, and a dot plot was made, and a truncation was made at the position where the slope change was obvious, and then the 43 samples were divided into a high group and a low group (among which, 17 patients were low and 26 patients were high according to OS, and 27 patients were low and 16 patients were high according to PFS). The mIHC results from each patient in the groups (including the percentage and density of each type of cell in the tumor and mesenchymal area) were analyzed and the p-value was obtained by using the wilcoxon rank-sum test and the grouping box plots.

Survival analysis involved the generation of PFS and OS curves and their respective 95% confidence interval (CI) using the Kaplan-Meier method, with comparisons conducted using the log-rank test. Hazard ratios (HRs) were computed and compared using univariate Cox proportional hazards regression models. The safety population included all patients who had received at least one dose of the study medications and had safety records. Safety was analyzed mainly using descriptive statistics. All statistical analyses were performed using R software (v4.1.2). A two-tailed P-value ≤ 0.05 was considered statistically significant unless indicated otherwise. More details can be seen in the Supplemental data I.

Results

Patient baseline characteristics

Between March 2022 and September 2022, 53 patients were screened for eligibility. 46 were eligible for enrollment and were included in the ITT population (Fig. 1). All 46 patients received at least one dose of the study medication and were included in the FAS and the safety set (SS). At the data cutoff date (February 29, 2024), treatment was ongoing in 15 patients (32.6%). 31 patients discontinued the treatment and twelve patients (12/31, 38.7%) went on to receive additional anti-tumor therapy, including immune therapy plus chemotherapy ($n = 4$), chemotherapy ($n = 2$), radiotherapy ($n = 2$) and anlotinib therapy ($n = 2$), and immune therapy plus nimotuzumab ($n = 1$) and interventional therapy ($n = 1$).

The median age was 67 years (range 44–77) and 31 patients (67.4%) were male. Most patients (45/46, 97.8%) had stage IVA ($n = 9$) or IVB disease ($n = 36$). Fifteen patients (32.6%) had lung metastases and seven (15.2%) had liver metastases, and 27 (58.7%) had metastases to more than two sites. Patient demographic and baseline characteristics are described in Table 1.

Clinical efficacy

At the data cutoff date, the median follow-up time was 17.64 months (IQR, 10.71–18.60 months). One patient achieved CR and 25 attained PR as the best overall response assessed by investigators per RECIST version 1.1 (Table 2). The median time to response was 1.46 months (IQR, 1.39–2.95 months). The investigators-confirmed ORR was 56.5% (95% CI 41.1–71.1). 16 patients had SD and the DCR was 91.3% (95% CI 79.2–97.6). One patient developed PD (Fig. 2A and B). The median DOR was 17.81 months (95% CI 12.68–20.60), and 82.4% patients responded to treatment at 12 months (Fig. 2C and D, Supplemental Table 1).

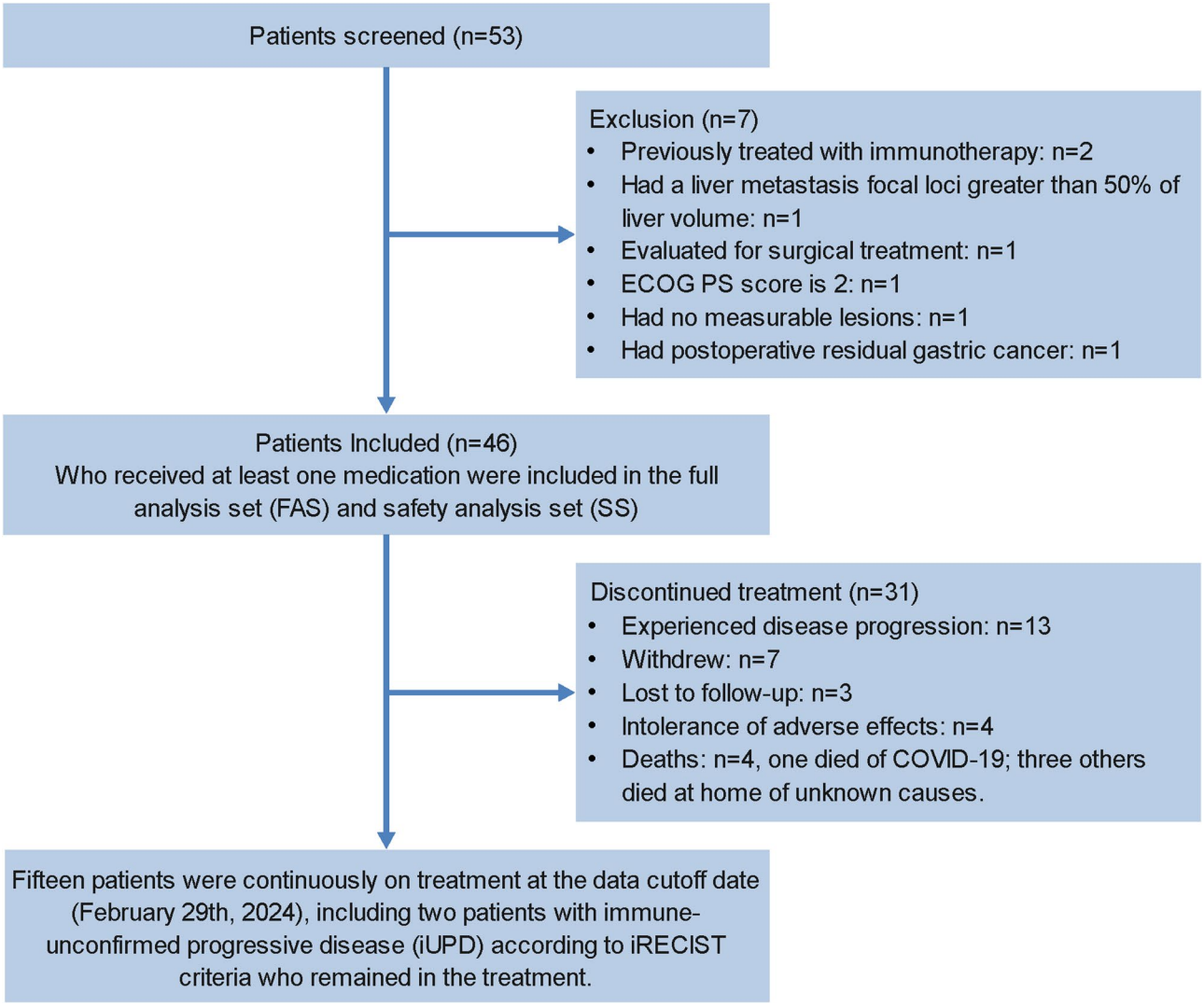

Fig. 1 Patient disposition chart

For survival outcomes, 21 PFS events occurred per RECIST version 1.1. The median PFS was 15.74 months (95% CI 9.03–21.91, Fig. 2E). The PFS rate was 58.8% (95% CI 41.0-72.9) at 12 months and 43.9% (95% CI 26.0-60.4) at 18 months (Supplemental Table 1). The median OS was 20.57 months (95% CI 14.65- not estimable [NE], Fig. 2F). Twenty-two patients died. The OS rate was 71.7% (95% CI 56.4–82.5) at 12 months and 51.6% (95% CI 35.4–65.6) at 18 months (Supplemental Table 1).

Exploratory subgroup analysis showed that patients with liver metastases had a shorter median DOR (5.49 months, 95% CI NE) than patients without liver metastases (17.81 months, 95% CI 16.53–20.60, $P<0.001$, Supplemental Table 2). Meanwhile, the benefit of anlotinib plus benmelstobart therapy with respect to ORR was homogeneous across clinical subgroups of advanced ESCC patients (Supplementary Table 2). Subgroup analysis showed no difference in survival outcomes (Supplementary Table 3), except for a significantly longer median PFS in patients without liver metastases than those with liver metastases (17.91 months, 95% CI 9.63–21.91 versus 6.80 months, 95% CI 2.73–6.90, $P<0.001$) and a longer median OS in patients with an ECOG performance status score of 0 versus 1 (not reached, 95% CI 11.60-NE versus 17.81 months, 95% CI 10.41-NE; $P=0.046$).

Safety

The dose of anlotinib was adjusted for 6 patients (13.0%), changing from 12 mg to 10 mg, with one patient needing a further reduction to 8 mg. Benmelstobart treatment was paused in three patients (6.5%). Additionally, four patients (8.7%) discontinued both anlotinib and benmelstobart treatments. TRAEs occurred in 43 patients (93.5%) while grade 3 or higher TRAEs occurred in 13 patients (28.3%). Any-grade TRAEs occurring in ≥ 30.0%

Table 1 Patient demographic and baseline characteristics

Characteristic	Anlotinib plus benmelstobart (*n* = 46)
Age, years	
Median (Range)	67 (44–77)
< 65	15 (32.6)
≥ 65	31 (67.4)
Sex	
Male	31 (67.4)
Female	15 (32.6)
ECOG performance status score[a]	
0	14 (30.4)
1	32 (70.6)
Stage of disease[b]	
III	1 (2.2)
IVA	9 (29.6)
IV B	36 (78.3)
Prior surgery	
Yes	19 (41.3)
No	27 (58.7)
Median target lesion size (range), mm[c]	27.3 (10.1-246.8)
Number of metastases	
≤ 2	19 (41.3)
> 2	27 (58.7)
Sites of metastases	
Lungs	15 (32.6)
Liver	7 (15.2)
Lymph nodes	25 (54.4)
Others	27 (58.7)

Data are expressed as number (%) unless otherwise indicated. The percentage may be higher than 100% due to rounding

[a]ECOG performance status scores range from 0 to 5, with 0 indicating no symptoms and higher scores indicating greater disability

[b]American Joint Committee on Cancer (AJCC) stage

[c]Tumor size is the sum of the largest diameters of all target lesions (shortest diameter for lymph nodes)

Table 2 Best overall tumor response of investigator-evaluation

Response	Investigators-confirmed (*n* = 46)
CR, n (%)	1 (2.2)
PR, n (%)	25 (54.4)
SD, n (%)	16 (34.8)
PD, n (%)	1 (2.2)
NE, n (%)	3 (6.5)
ORR, n (%, 95%CI)	56.5 (41.1, 71.1)
DCR, n (%, 95%CI)	91.3 (79.2, 97.6)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, inevaluable; ORR, objective response rate; DCR, Disease Control Rate

of the patients included hypertension (47.8%), hypothyroidism (37.0%), white blood cell count decreased (34.8%) and anemia (32.6%) (Table 3). Hypertension was the most frequent grade 3 or higher TRAE (10.9%) followed by hyponatremia (4.3%) and neutrophil count decreased (4.3%). Grade 3 or higher proteinuria, palmar-plantar erythrodysesthesia syndrome and upper gastrointestinal hemorrhage each occurred in 1 patient (2.2%). Grade 2 esophageal fistula occurred in 1 patient (2.2%). No treatment-related death was reported.

Genomic alterations and clinical efficacy

The NGS was performed on baseline tumor tissue samples from 41 participants available for further exploration of potential biomarkers to predict response. Among these patients, 22 patients were responders (CR or PR) and 17 were non-responders (SD or PD). Sequencing data analysis showed that all the patients (100.0%, 41/41) harbored gene mutations. The identified mutated genes included known ESCC drivers such as *TP53* (85.4%), *PIK3CA* (31.7%), and *CDKN2A* (29.3%), *LRP1B* (29.3%), and *CREBBP* (22.0%). Other cancer-related genes included *FAT1* (14.6%) and *NOTCH3* (9.8%) (Fig. 3A). The overall TMB ($P = 0.200$) or APOBEC-TMB ($P = 0.110$) was not significantly associated with tumor response or survival outcomes (Supplemental Fig. 1).

Genes exhibiting mutations in more than three patients (≥ 3) were chosen for further analysis to assess their predictive value on patient outcomes. The results suggested that mutations in three genes, *TP53*, *FAT1* and *NOTCH3*, had association with the clinical efficacy. Patients with *TP53* mutations exhibited numerically higher objective response rates (63.6% vs. 16.7%, $P = 0.068$) and demonstrated clinically meaningful improvements in survival outcomes, though only the OS difference reached statistical significance (median PFS: 15.74 vs. 5.91 months, $P = 0.084$; median OS: not reached vs. 10.92 months, $P = 0.018$; Fig. 3B). While not reaching statistical significance, patients with *FAT1* gene mutation also achieved numerically higher ORR (100.0% versus 51.4%, $P = 0.118$) and longer PFS (median PFS: 20.58 versus 9.92 months, $P = 0.089$). A significant overall survival benefit was observed (median OS: not reached [NR] versus 17.81 months, $P = 0.032$, Fig. 3C). In contrast, Patients harboring *NOTCH3* mutations had significantly lower ORR (0.0% versus 62.9%, $P = 0.029$), shorter PFS (median PFS: 3.68 versus 17.91 months, $P = 0.002$) and shorter OS (median OS: 4.73 months versus NR, $P = 0.026$, Fig. 3D).

To investigate the role of immune cells in ESCC patients treated with anlotinib plus benmelstobart, the tumor microenvironment (TME) composition was examined using mIHC (Supplemental Fig. 2), MCP-counter analysis and GSEA. While panel-1 markers were not significantly associated with PFS or OS, CD23 density and percentage in tumor and mesenchymal regions showed significant association with PFS (Supplemental Fig. 3A and 3B). Patient harboring mutated *TP53* had greater infiltrations of CD23 + cells ($P = 0.029$) and CD21 + cells ($P = 0.034$),

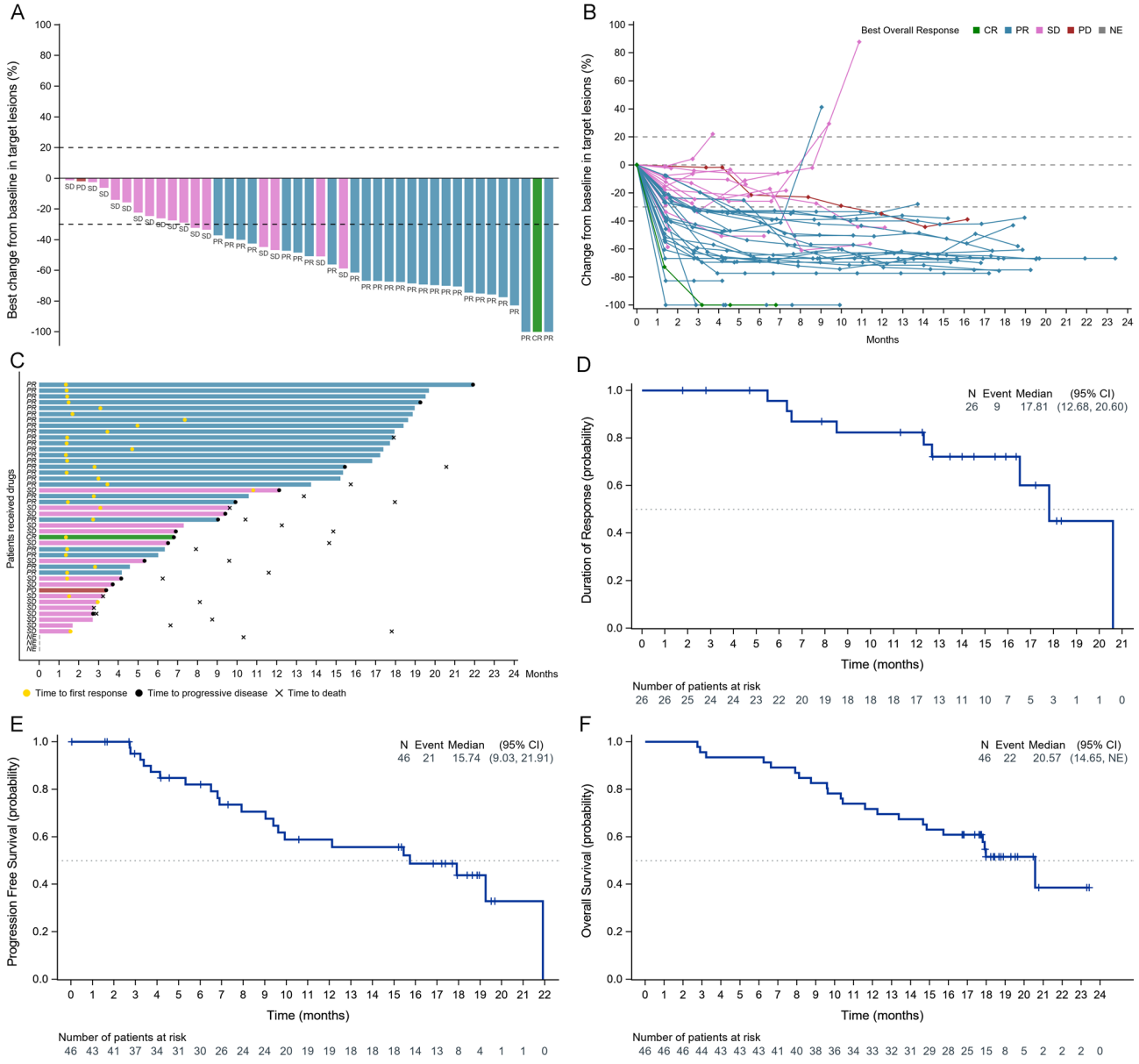

Fig. 2 Treatment responses and survival outcomes. (**A**) Waterfall plots of the best percentage changes for the sum of target lesion diameters are shown for individual patients with advanced, recurrent or metastatic esophageal squamous cell carcinoma (ESCC) assessed by investigators per RECIST version 1.1. The green dotted line indicates a 30% reduction and the orange dotted line indicates a 20% increase in the target lesion size. Each bar represents one patient in the intention-to-treat (ITT) population. (**B**) The spider plot of individual changes in the sum of unidimensional tumor measurements over time relative to baseline tumor burden. (**C**) Swimmer plots of time to tumor response (months) of individual patients. Each swim lane represents one patient in the ITT population. CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease. (**D**) The Kaplan-Meier curves of duration of response (DOR). (**E**) The Kaplan-Meier curves of progression-free survival (PFS) of advanced, or metastatic ESCC patients in the ITT population. (**F**) The Kaplan-Meier curves of overall survival (OS)

increased tumor-associated fibroblasts ($P = 0.019$), upregulated INF-gamma and alpha responses and downregulated EMT (Supplemental Fig. 4A). Those with mutated *FAT1* showed greater infiltrations of CD23+ ($P = 0.019$) and CD20+/KI67 + cells ($P = 0.037$), higher cytotoxicity scores ($P = 0.025$), upregulation of G2M checkpoint and downregulation of INF-gamma response (Supplemental Fig. 4B). In contrast, patients with mutated *NOTCH3* demonstrated lower infiltrations of bone marrow-derived DCs ($P = 0.050$), monocytes ($P = 0.008$) and macrophage ($P = 0.008$) in the TME, but no significant pathway enrichment (Supplemental Fig. 4C).

We further utilized mutated *TP53+/FAT1+/NOTCH3-* as a predictive signature for the study cohort (Fig. 4A). In this cohort, we compared the clinical efficacy of combined therapy in the patients carrying only *TP53* or *FAT1*

Table 3 Treatment-related adverse events (TRAEs) in all patients with all grades (occurring in ≥ 5% of patients) and ≥ grade 3

TRAEs	Any grade (*n*, %)	Grade 3 or higher (*n*, %)
All	43 (93.5)	13 (28.3)
Hypertension	22 (47.8)	5 (10.9)
Hypothyroidism	17 (37.0)	0
White blood cell decreased	16 (34.8)	1 (2.2)
Anemia	15 (32.6)	0
Hyperthyroidism	11 (23.9)	0
Platelet count decreased	11 (23.9)	1 (2.2)
Neutrophil count decreased	10 (21.7)	2 (4.3)
Hypoalbuminemia	9 (19.6)	0
Malaise	9 (19.6)	0
Hypokalemia	7 (15.2)	0
Palmar-plantar erythrodysesthesia syndrome	7 (15.2)	1 (2.2)
Proteinuria	7 (15.2)	1 (2.2)
Hyponatremia	6 (13.0)	2 (4.3)
Diarrhea	5 (10.9)	0
Thyroid stimulating hormone increased	5 (10.9)	0
Aspartate aminotransferase increased	4 (8.7)	0
Constipation	4 (8.7)	0
Abdominal distension	3 (6.5)	0
Alanine aminotransferase increased	3 (6.5)	0
Anorexia	3 (6.5)	0
Lymphocyte count decreased	3 (6.5)	1 (2.2)
Mucositis oral	3 (6.5)	0
Nausea	3 (6.5)	0
Sinus bradycardia	3 (6.5)	0
Upper gastrointestinal hemorrhage	3 (6.5)	1 (2.2)
Toothache	2 (4.3)	1 (2.2)

Toxicities were assessed using the Common Toxicity Standards of the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTC AE) version 5.0

mutations but without *NOTCH3* alterations (*TP53+/FAT1+/NOTCH3-*, $n = 32$, Supplemental Table 4) and the rest of patients ($n = 9$). A significantly greater proportion of patients bearing the predictive signature showed ORR to combined antiangiogenic and immune therapy than those without the mutational signature (65.6% versus 11.1%, $P < 0.001$) (Fig. 4B). Patients bearing the predictive mutational signature had a significantly longer median PFS (17.91 versus 5.32 months), with a 73.0% reduction in the risk of progression or death (HR = 0.27, $P = 0.005$) (Fig. 4C). The median OS was not reached for patients with the predictive signature, compared to 9.59 months for those without (HR = 0.29, $P = 0.006$) (Fig. 4D). In addition, we performed multivariate Cox analyses adjusting for liver metastasis and ECOG score (identified as significant covariates in univariate analysis). As detailed in the Supplemental Tables 5 and 6, the *TP53+/FAT1+/NOTCH3-* signature remained an independent predictor for both PFS (HR 0.18, 95% CI 0.06–0.51, $P = 0.001$) and OS (HR 0.38, 95% CI 0.14–0.99, $P = 0.047$) after multivariable adjustment.

mIHC demonstrated greater infiltrations of CD21 + cells ($P = 0.022$) and CD20 + KI67 + cells ($P = 0.037$, Fig. 4E) in the TME in patients with the predictive signature. MCP-counter analysis of TCGA data revealed significantly greater infiltrations of myeloid DCs ($P = 0.029$) in the TME in patients with the *TP53+/FAT1+/NOTCH3-* mutational signature ($n = 79$, Fig. 4F). GSEA showed upregulation of EMT and downregulation of IL6-JAK-STAT3, IFN-α pathways and apoptosis in this subgroup of patients (Fig. 4G).

PD-L1 expression and clinical efficacy

Among 42 patients with PD-L1 CPS or TPS results, no significant differences were found in ORR, PFS, or OS between CPS ≥ 1 and CPS < 1 groups. Similarly, for TPS, no significant differences were observed in ORR, PFS, or OS between TPS ≥ 1% and TPS < 1% groups (Supplemental Fig. 5).

Discussion

To our knowledge, this is the first clinical study of a chemo-free regimen combining anlotinib with benmelstobart in advanced ESCC. The regimen showed promising efficacy, achieving an ORR of 56.5%, a median PFS of 15.74 months, and a median OS of 20.57 months. These outcomes significantly outperform traditional frontline chemotherapy [7, 8] and compare favorably with immune checkpoint inhibitors plus chemotherapy. Recent trials of PD-1/PD-L1 inhibitors combined with chemotherapy reported ORRs ranging from 45 to 69.3%, median PFS from 5.7 to 7.3 months, and median OS from 12.4 to 17 months [9]. While our ORR falls within this range, the extended PFS and OS suggest a more durable response with our chemo-free approach.

The extended PFS and OS may be due to the unique mechanism of action of our combination therapy. However, patient factors like PD-L1 expression and prior treatments could also influence outcomes. The NATION-1 trial indicated that single-agent immunotherapy improves recurrence-free survival (RFS) and OS after surgery for resectable ESCC [34], suggesting that chemotherapy may not be necessary for some patients. Our findings further support a chemo-free approach as a potent yet less toxic alternative to current standard-care regimens. In the liver metastasis subgroup, both DOR and PFS were poorer. This may be due to the inherently worse prognosis of patients with liver metastases and their potential insensitivity to immunotherapy [35, 36]. Additionally, we noted significant differences in subgroup sizes and baseline characteristics between the two groups, which could also contribute to the poorer outcomes in the liver metastasis subgroup.

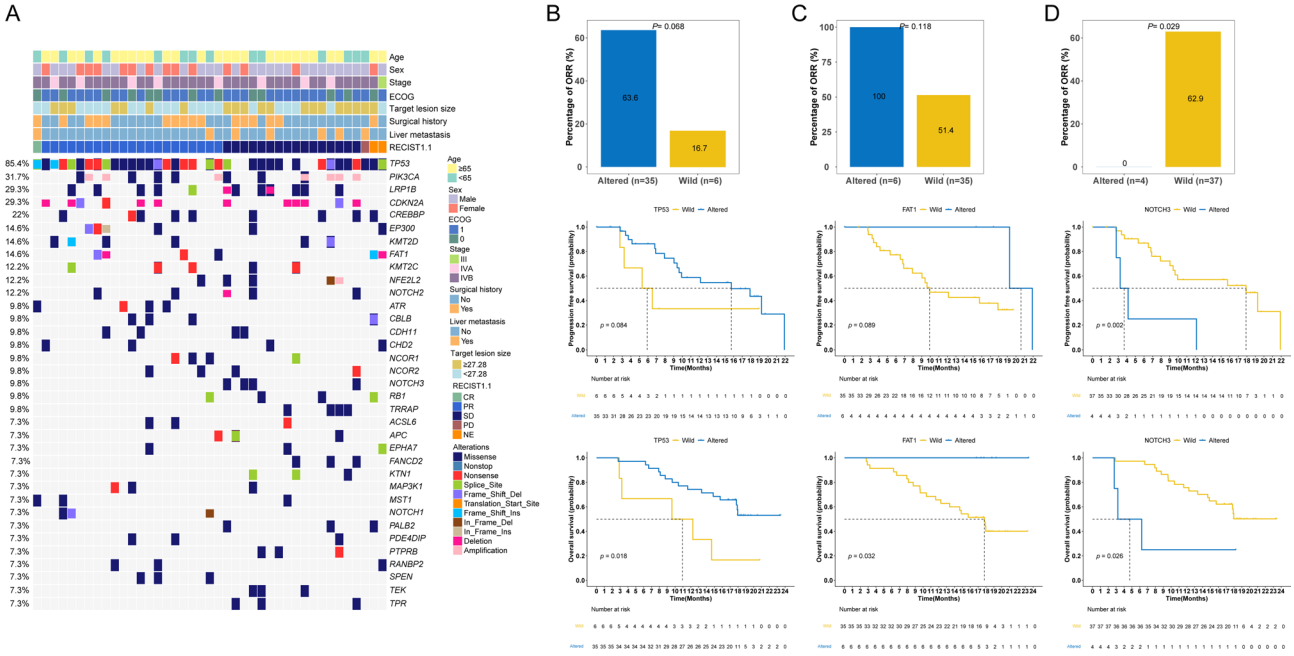

Fig. 3 Somatic genomic alterations and tumor responses. (**A**) OncoPrint of functional driver mutations of most frequent somatic mutations and gene copy number alterations identified in 41 ESCC samples. Mutation types and gene copy number alterations are color-coded according to the legend. Rows represent genes, and columns represent samples. Glyphs and color coding are used to summarize distinct genomic alterations including mutations, copy number alterations (amplifications and homozygous deletions), and best response. (**B**) *TP53* gene mutation was associated with higher ORR, longer PFS or OS. (**C**) *FAT1* gene mutation was associated with higher ORR, longer PFS or OS. (**D**) *NOTCH3* mutations were only detected in the non-responders and were associated with shorter PFS or OS

Immunochemotherapy combinations show survival advantages over doublet chemotherapy but are associated with significant renal and hepatic toxicities [10–12]. In contrast, our chemo-free regimen had an acceptable toxicity profile, with grade 3 or higher treatment-related adverse events (TRAEs) occurring in 28.3% of patients. This rate is lower than other dual immune inhibition and VEGF inhibition regimens [10, 17] and immune checkpoint inhibition plus chemotherapy combinations (ranging from 49 to 72% in first-line ESCC treatments) [9, 37, 38]. No treatment-related deaths were reported, suggesting a more favorable safety profile while maintaining efficacy.

The biomarker analysis further revealed a novel mutational signature involving *TP53*, *FAT1*, and *NOTCH3* genes that predicts response to this combination therapy in ESCC. Patients with *TP53* or *FAT1* mutations but lacking *NOTCH3* mutations (*TP53+/FAT1+/NOTCH3-*) showed significantly improved outcomes, including a 73.0% reduction in the risk of progression or death. This finding could have important implications for patient selection and personalized treatment strategies.

TP53 mutations are highly prevalent in ESCC [24], and the ORR of first-line platinum-based chemotherapy for esophageal cancer is limited [15]. Additionally, tumors with *TP53* mutations generally respond poorly to immunotherapy [39], which accounts for the minimal OS benefit observed with first-line immune-combination chemotherapy in advanced ESCC [15]. Our findings demonstrate that *TP53*-mutated tumors exhibit upregulation of interferon-α/γ pathways and apoptotic signaling, potentially enhancing immune-mediated tumor clearance, while showing downregulated EMT pathways associated with reduced metastatic potential [40]. The observed increased infiltration of dendritic cells, T cells, and B cells aligns with prior evidence that *TP53* mutations elevate immune checkpoint expression and activate Teff/IFN-γ signaling [41]. *TP53*-mutated malignancies demonstrate heightened VEGF-mediated angiogenesis [42, 43], which may explain their particular susceptibility to anlotinib's dual mechanisms: (1) vascular normalization to facilitate immune cell infiltration, and (2) synergy with PD-L1 blockade through adaptive immune gene induction [44, 45]. In addition, VEGF inhibitors have shown greater efficacy against *TP53* mutations [46, 47]. Therefore, incorporating VEGF inhibitors for *TP53*-mutated ESCC is crucial.

FAT1 is a tumor suppressor gene involved in Wnt signaling, and its mutations have been linked to increased T-cell infiltration in some cancers. As a critical regulator of cell adhesion and polarity [48–50], *FAT1* mutations demonstrate cancer-type-specific immunomodulatory effects - increasing CD8 + T/NK cells in our ESCC cohort while associating with M1 macrophage polarization

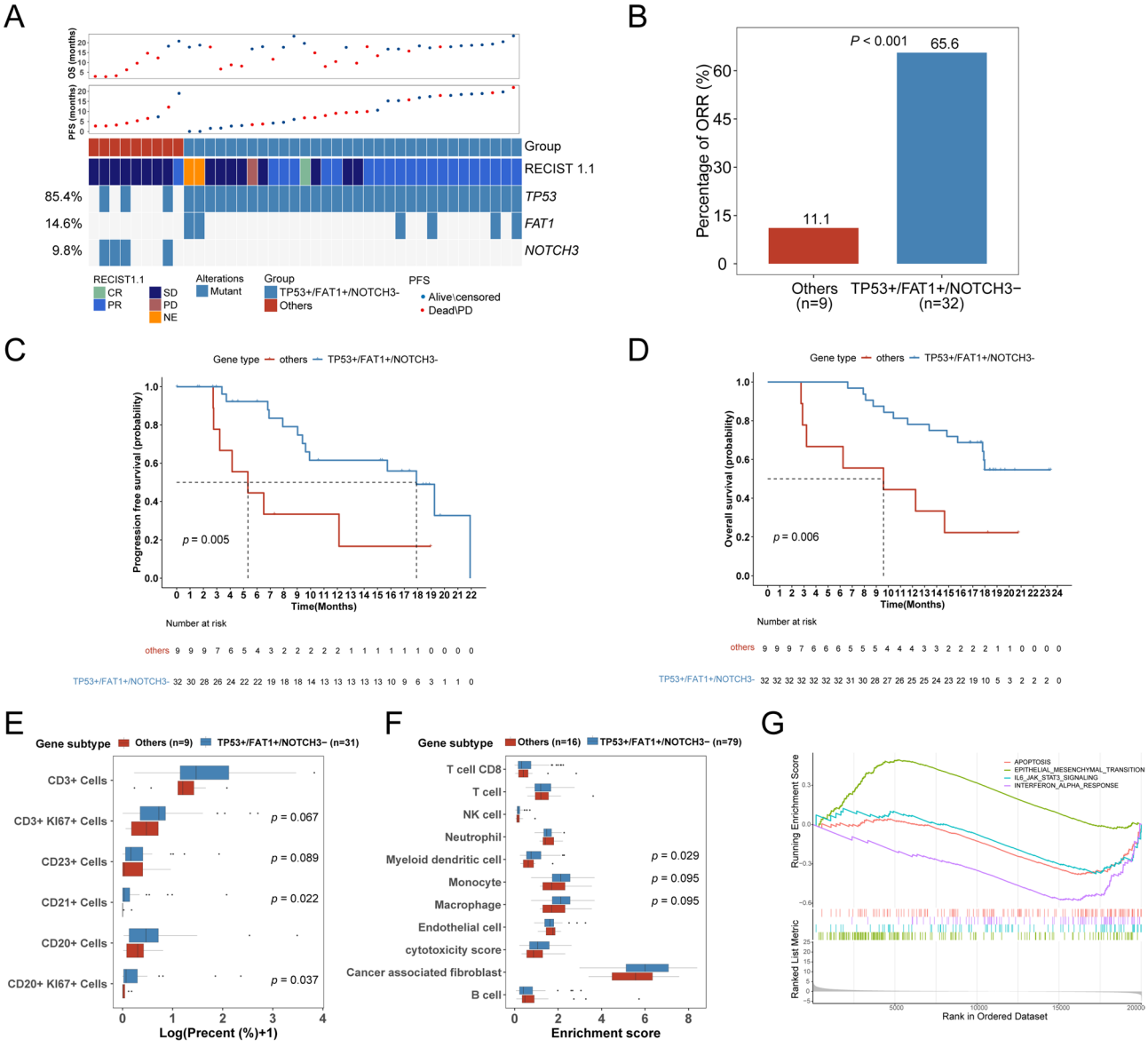

Fig. 4 A *FAT1+/TP53+/NOTCH3-* predictive mutational signature in ESCC patients treated with benmelstobart plus anlotinib. (**A**) OncoPrint of *TP53*, *FAT1* and *NOTCH3* mutations 41 patients. Patients with *TP53+/FAT1+* mutations but without *NOTCH3* mutations ($n = 32$) had significantly higher ORR (**B**), longer PFS (**C**) or OS (**D**). mIHC showed greater infiltrations of CD21 + and CD20+/Ki67 + cells, as well as CD23 + cells and CD3+/KI67 + cells in the TME in patients with the predictive signature (**E**). MCP-counter analysis showed greater infiltrations of myeloid DCs, monocytes and macrophage cells in the TME in patients with the *TP53+/FAT1+/NOTCH3-* mutational signature in TCGA ESCC cohort (**F**). GSEA showed upregulation of EMT and downregulation of IL6-JAK-STAT3, IFN-α and apoptosis pathways in this subgroup of patients in TCGA ESCC cohort (**G**)

in melanoma [51]. Mechanistically, *FAT1* loss induces hybrid EMT states [52] while disrupting vascular homeostasis via YAP/TAZ dysregulation, creating a microenvironment where anlotinib's antiangiogenic effects may optimally complement PD-L1 inhibition's immunostimulatory actions.

Conversely, *NOTCH3* mutations are associated with poorer outcomes. NOTCH signaling plays a complex role in cancer progression and immune regulation [53], and our findings suggest that *NOTCH3* mutations may confer resistance to this combination therapy in ESCC, consistent with gastric cancer data linking *NOTCH3* to immunosuppressive M2/Treg dominance [54]. This suggests *NOTCH3* mutations may establish an immune-evasive niche that resists combination therapy [55, 56]. This observation warrants further investigation into the specific mechanisms by which *NOTCH3* mutations might influence treatment response.

In addition, the mIHC analysis and MCP-counter analysis confirmed greater infiltrations of myeloid dendritic cells, monocytes, and macrophages in the TME of patients with the *TP53+/FAT1+/NOTCH3-* mutational

signature. Our observation of increased B cell infiltration in tumors with the predictive signature suggests a potential mechanism for enhanced immune response in these patients [51, 57–59]. These findings further suggest that the identified genetic alterations may create a more immunologically active tumor microenvironment, potentially enhancing the efficacy of immunotherapy. The *TP53+/FAT1+/NOTCH3-* signature appears to define a biological triad where: (1) *TP53/FAT1* mutations collectively promote immunogenic microenvironments, (2) *NOTCH3* deficiency counteracts these effects, and (3) anlotinib selectively exploits the resultant vascular/immune dysregulation. Future studies should delineate their crosstalk within Wnt/Notch/p53 networks using preclinical models.

We found no significant association between PD-L1 expression and clinical outcomes, contrasting with some previous studies but aligning with the limitations of PD-L1 as a single biomarker for immunotherapy response [10, 60]. This lack of association may be due to the unique mechanism of action of our combination therapy, which could be less dependent on baseline PD-L1 expression compared to single-agent PD-1/PD-L1 inhibitors [61–63]. Furthermore, the unique mechanism of our combination therapy may explain PD-L1's diminished predictive value. Our regimen combines anlotinib (an anti-angiogenic agent) that remodels the tumor vascular microenvironment to facilitate immune cell infiltration [44, 45] with benmelstobart. This synergistic approach likely operates through multiple pathways rather than relying solely on baseline PD-L1 expression, thereby reducing PD-L1 (CPS/TPS) value as a standalone predictive biomarker. Similar observations of PD-L1's limited predictive utility have been reported in ESCC studies combining immunotherapy with chemotherapy [64]. While both represent combination immunotherapies, the mechanistic differences between chemotherapy and our anti-angiogenic approach suggest that PD-L1 expression may indeed lack significant value for identifying beneficiary populations in combination immunotherapy settings.

The study has several limitations. Firstly, this is a single-arm trial without a control arm. A randomized controlled trial (e.g., comparing immunotherapy + chemotherapy with our regimen) should be conducted to validate whether this signature truly predicts additional benefits from the combination of immunotherapy and anti-angiogenic therapy. Secondly, the sample size in this study is small. While rigorous statistical methods were applied, the sample size disparity between subgroups may affect result robustness, warranting validation in larger cohorts. Additionally, a more comprehensive biomarker signature may be needed to identify patients who will benefit.

In conclusion, the anlotinib-benmelstobart regimen demonstrated clinically meaningful activity and acceptable safety as first-line therapy for advanced ESCC. Exploratory analyses suggest that patients with the *TP53+/FAT1+/NOTCH3-* signature may derive greater benefit from treatment, but these preliminary observations require confirmation in prospective, controlled studies. These findings support further investigation of this chemo-free combination in biomarker-defined populations.

Abbreviations

AEs	Adverse events
CR	Complete response
CPS	Combined positive score
CI	Confidence interval
DCR	Disease control rate
DOR	Duration of response
ESCC	Esophageal squamous cell carcinoma
FFPE	Formalin-fixed and paraffin-embedded
FAS	Full analysis set
ITT	Intention-to-treat
mIHC	Multiplex immunohistochemistry
NGS	Next-generation sequencing
NE	Not estimable
NR	Not reached
ORR	Objective response rate
OS	Overall survival
PFS	Progression-free survival
PR	Partial response
PD	Progressive disease
PD-L1	Programmed death ligand 1
SD	Stable disease
SS	Safety set
TRAEs	Treatment-related adverse events
TMB	Tumor mutation burden
TPS	Tumor proportion score

Supplementary Information

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Supplementary Material 1

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Author contributions

F.W., X.M., X.Y., Y.H., and W.W. conceived and designed the study. F.W., X.M., X.Y., Y.H., Z.Z., J.X., Y.C., Y.Z., T.L., M.S., Z.S., T.W., W.W., L.S., L.G., M.M., and L.W. performed data collection. F.W., X.M., X.L., D.X., Y.M., G.J., Y.Q., B.J., D.Z., B.H., X.W., and Z.P. performed the data analysis and contributed to the interpretation of the data. All authors wrote the original manuscript. All authors reviewed and approved the manuscript.

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None.

Data availability

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the ethics committee of The First Affiliated Hospital of Zhengzhou University (NO: L2021-Y277-002), The First Affiliated Hospital of Nanyang Medical College (NO: 2021-040-02), Anyang Tumor Hospital (NO: 2022-03-002-H01), The First Affiliated Hospital of Henan University of Science and Technology (NO: 2022-0033), and Zhumadian Central Hospital (NO: 2022-01-002H01). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from individual participants.

Consent for publication
Written informed consent was obtained from individual participants.

Competing interests
The authors declare no competing interests.

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