

Anlotinib as Third-Line or Later Therapy in Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma: Real-World Efficacy and Safety Outcomes

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Background: Anlotinib, a multi-targeted tyrosine kinase inhibitor, has demonstrated anti-angiogenic and immunomodulatory activity in several solid tumors; however, its efficacy and predictive biomarkers in recurrent or metastatic head and neck squamous cell carcinoma (R/M HNSCC) remain unclear.

Methods: This retrospective, single-center study included 68 patients with histologically confirmed R/M HNSCC who received anlotinib as third-line or later therapy (following failure of at least two prior systemic lines; 12 mg once daily on days 1–14 every 21 days, with dose reductions to 10 or 8 mg as needed) between January 2021 and October 2023. Tumor response was evaluated according to RECIST v1.1 in patients with available radiologic follow-up. Survival outcomes were analyzed in the overall treated population. Archived tumor tissues were subjected to next-generation sequencing (NGS) and multiplex immunofluorescence (mIF) to exploratorily assess genomic alterations and immune microenvironment features.

Results: Among 68 treated patients, 14 achieved partial response, and 39 had stable disease, yielding an objective response rate (ORR) of 22.1% and a disease control rate (DCR) of 74.6% in treated patients. The median progression-free survival (PFS) and overall survival (OS) in the overall cohort were 6.3 and 8.4 months, respectively. Patients with oropharyngeal carcinoma and ECOG performance status 0–1 demonstrated improved outcomes. NGS analysis identified frequent alterations in TP53, PIK3CA, CDKN2A, PTEN, and FGF/FGFR pathways. PI3K pathway alterations were not associated with prolonged PFS. Tumors with PD-L1 CPS ≥ 1 and higher CD8⁺ T-cell infiltration exhibited an inflamed phenotype and were associated with improved response. Grade ≥ 3 adverse events occurred in 25.0% of patients, most commonly hypertension and hand-foot syndrome.

Conclusion: Anlotinib demonstrated promising responses with manageable toxicity in a heavily pretreated R/M HNSCC population. Integration of genomic and immune microenvironment features may provide hypothesis-generating insights into patient selection.

Plain Language Summary:

1. Why was the study done?

Patients with advanced head and neck cancer has returned or spread often have few options after two or more prior treatments. Researchers tested a drug called anlotinib in this difficult-to-treat group.

2. What did the researchers do and find?

The study included 68 patients whose cancer had progressed after at least two earlier treatments. Anlotinib stopped tumor growth or shrank tumors in 78 out of 100 patients (disease control rate 78.0%). Tumors shrank significantly in about 20 out of 100 patients (response rate 20.5%). On average, patients lived for 8.4 months, and their cancer did not worsen for 6.3 months. Patients with tumors that had higher levels of a marker called PD-L1 (linked to immunotherapy response) tended to do better. Serious side effects occurred in 25% of patients; the most common were high blood pressure and hand-foot skin reactions.

3. What do these results mean?

Anlotinib shows promise as a later-line treatment for advanced head and neck cancer, with manageable side effects. The study also suggests that PD-L1 levels and certain immune features might help predict which patients benefit most.

Keywords: anlotinib, head and neck squamous cell carcinoma, tyrosine kinase inhibitor, third-line therapy, immunotherapy resistance

Introduction

Head and neck squamous cell carcinoma (HNSCC) comprises a diverse group of aggressive epithelial tumors arising from the mucosal linings of the oral cavity, oropharynx, larynx, and hypopharynx.¹ HNSCC is biologically heterogeneous across different anatomical subsites, and notably, oropharyngeal tumors, which are often associated with human papillomavirus (HPV), generally exhibit a more favorable prognosis and distinct immune characteristics than other HNSCC subtypes.² Despite significant advances in multimodal treatment strategies, the prognosis for patients with recurrent or metastatic (R/M) HNSCC remains poor, with median overall survival (OS) following standard therapies rarely exceeding 12 months, highlighting the limited efficacy of currently available treatments in this setting.^{1,3}

Over the past decade, the therapeutic landscape for R/M HNSCC has undergone notable changes, particularly with the introduction of immune checkpoint inhibitors (ICIs), which have marked a shift in the management paradigm. Despite these advances, both primary and acquired resistance to immunotherapy pose major barriers, with approximately 60% of patients exhibiting no response to monotherapy with PD-1/PD-L1 inhibitors.⁴ In clinical practice, first-line treatment for R/M HNSCC generally includes platinum-based chemotherapy in combination with either cetuximab or pembrolizumab, guided by PD-L1 expression levels.⁵ For patients who have not previously received immunotherapy, single-agent ICIs such as nivolumab or pembrolizumab serve as second-line options, yielding objective response rates (ORRs) of approximately 13–18%.⁶ However, once disease progression occurs after both platinum-based regimens and ICIs, treatment choices become limited and largely palliative. Commonly used agents in this setting include taxanes, methotrexate, and cetuximab-based regimens; however, these approaches are associated with modest response rates, typically below 15–20%,⁷ and a median progression-free survival of approximately 2–3 months.⁸ These outcomes underscore the absence of an effective standard of care in the post-platinum/ICI setting and highlight the need for novel therapeutic strategies.

Apart from its role in tumor angiogenesis, vascular endothelial growth factor (VEGF) signaling contributes to an immunosuppressive tumor microenvironment by inhibiting dendritic cell maturation, promoting regulatory T cell expansion, and impairing cytotoxic T-cell infiltration.⁹ These effects have been implicated in both primary and acquired resistance to immune checkpoint blockade.¹⁰ In this context, anti-angiogenic agents may exert immunomodulatory effects by normalizing tumor vasculature and alleviating VEGF-mediated immune suppression, thereby potentially restoring antitumor immune responses; providing a rationale for continued activity of anti-angiogenic tyrosine kinase inhibitors even after progression on PD-1/PD-L1 inhibitors.¹¹ Recent investigations have highlighted the role of tumor angiogenesis and immune evasion in mediating resistance to therapy in HNSCC.¹² One of the defining features of HNSCC is aberrant angiogenesis, largely driven by overexpression of VEGF and its receptors.¹³ Moreover, beyond promoting neovascularization, VEGF contributes to an immunosuppressive tumor microenvironment by inhibiting dendritic cell maturation, enhancing regulatory T cell activity, and impairing tumor-infiltrating lymphocyte function.¹³ Together, these findings suggest that targeting VEGF-mediated pathways may enhance anti-tumor immunity and improve the effectiveness of immunotherapy in R/M HNSCC.

Anlotinib is an orally administered multi-target tyrosine kinase inhibitor (TKI) with potent inhibitory effects against VEGFR1-3, FGFR1-4, PDGFR α/β , c-Kit, and Met.¹⁴ Unlike the first-generation TKIs, anlotinib demonstrates a more favorable pharmacokinetic profile with a longer half-life, allowing once-daily dosing and potentially enhanced target inhibition.¹⁵ Preclinical studies have demonstrated that anlotinib exerts anti-tumor effects through multiple mechanisms, including inhibition of angiogenesis, direct antiproliferative activity, and modulation of the tumor immune microenvironment.¹⁶ In particular, anlotinib has been shown to enhance CD8⁺ T-cell infiltration and augment the efficacy of PD-1 blockade in murine models.¹⁷ Clinical data further support the therapeutic value of anlotinib in R/M HNSCC. A multicenter Phase II trial reported an ORR of 15.4%, with a median progression-free survival (PFS) of 5.9 months and manageable toxicity in patients previously treated with standard therapies.¹⁵ Real-world studies have corroborated these

findings, demonstrating sustained disease control even in tumors with low PD-L1 expression, which are generally less responsive to immunotherapy.¹⁶ Retrospective analyses indicate that anlotinib may achieve median OS durations of 7–9 months in the post-second-line treatment setting, surpassing historical outcomes associated with conventional cytotoxic agents or best supportive care.^{16,17}

Beyond HNSCC, the efficacy of anlotinib has been validated in other solid tumors. In the Phase III ALTER-0303 trial, anlotinib significantly prolonged OS compared with placebo in patients with advanced non-small cell lung cancer (NSCLC) receiving third-line therapy, while maintaining a favorable safety profile.¹⁸ Additionally, emerging evidence suggests that anlotinib may enhance responses to PD-1 inhibitors across multiple malignancies, including NSCLC, by reversing immune resistance.^{19,20} However, data on its application in R/M HNSCC, particularly following failure of immunotherapy, remain limited.

Given the dual angiogenic and immunomodulatory effects of anlotinib, we hypothesized that genomic alterations in angiogenesis-related pathways and features of the tumor immune microenvironment may influence therapeutic response; therefore, NGS and multiplex immunofluorescence were incorporated to explore potential predictive biomarkers. Herein, the present retrospective study assessed the real-world efficacy and safety of anlotinib in patients with R/M HNSCC who had progressed after at least two lines of systemic therapy, including platinum-based chemotherapy and PD-1/PD-L1 inhibitors. By evaluating tumor response, survival outcomes, and treatment-related adverse events, this study aimed to clarify the potential role of anlotinib as a post-second-line treatment option for this challenging patient population.

Methods

Study Design and Patients

This retrospective, single-center study assessed the data of patients with histologically confirmed R/M HNSCC who received anlotinib as post-second-line treatment setting, defined as treatment administered after failure of at least two prior systemic regimens, between January 2021 and October 2023 at our institution. The study protocol adhered to the ethical principles outlined in the Declaration of Helsinki and received approval from the institutional ethics committee. Due to the retrospective nature of the study and the use of anonymized patient data, the requirement for informed consent was waived.

Patient information was obtained from electronic medical records. These included demographic data, primary tumor site, pattern of metastasis, treatment history, and programmed death-ligand 1 (PD-L1) combined positive score (CPS), where available. Cases were considered eligible if they matched the following criteria: (1) age ≥ 18 years; (2) histologically confirmed R/M HNSCC with at least one measurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1; (3) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 2; (4) documented failure of at least two prior lines of systemic therapy (ie., post-second-line treatment), including both platinum-based chemotherapy and a PD-1/PD-L1 immune checkpoint inhibitor; (5) an estimated life expectancy of at least 3 months; and (6) adequate hematologic, renal, and hepatic function as determined by clinical laboratory evaluation.

In addition, cases with the following criteria were excluded: (1) presence of active or untreated central nervous system metastases; (2) uncontrolled hypertension despite appropriate medical management; (3) known coagulation disorders or active bleeding diatheses; or (4) prior treatment with anti-angiogenic TKIs.

Treatment Protocol

Anlotinib (Focovvi[®], Chia Tai Tianqing Pharmaceutical Group Co., Ltd, co Lianyungang City, Jiangsu Province, China) was administered orally as monotherapy, most commonly at an initial dose of 12 mg once daily for 14 consecutive days followed by a 7-day rest period in a 21-day cycle. For elderly patients (aged ≥ 75 years) or those with poor general condition, a lower starting dose of 10 mg was frequently selected at the discretion of the treating physician. Dose reductions to 10 mg or 8 mg were documented in cases of treatment-related adverse events (AEs), based on clinical judgment and individual patient tolerance. Treatment was generally continued until radiographic or clinical disease progression, emergence of unacceptable toxicity, patient refusal, or death.

Next-Generation Sequencing (NGS)

Archived formalin-fixed paraffin-embedded (FFPE) tumor samples collected at baseline from enrolled patients were subjected to targeted next-generation sequencing (NGS). DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and DNA concentration and purity were quantified with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation was performed using the KAPA HyperPlus Kit (Roche, Basel, Switzerland).

Sequencing was conducted on the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) using a hybrid-capture panel covering 520 cancer-related genes, including PIK3CA, TP53, CDKN2A, PTEN, EGFR, and members of the FGF/FGFR signaling pathway. The mean on-target sequencing depth exceeded 800×. Sequence alignment and variant calling were performed using BWA (v0.7.17) and GATK (v4.2), respectively. Variants were annotated against dbSNP, COSMIC, and ClinVar databases, and pathogenic or likely pathogenic variants were retained for downstream analyses.

Copy number alterations were inferred from normalized sequencing read depth. Homozygous deletion was defined as a $\log_2$ copy number ratio ≤ -1.0 , heterozygous loss as > -1.0 to ≤ -0.3 , and copy number gain/amplification as $\geq +0.3$. PTEN loss was defined specifically as copy number deletion (heterozygous or homozygous), while PTEN mutations were analyzed as a separate category. Alterations in the FGF/FGFR pathway were defined as genomic events involving FGFR1–4 and FGF ligands (eg., FGF3/4/19), including gene amplification, activating mutations, or gene fusions. In this cohort, no FGFR fusions were detected.

For variant filtering, a minimum variant allele frequency (VAF) threshold of 5% and a minimum sequencing depth of 100× at the variant locus were applied to ensure reliability. Variants with low base quality, significant strand bias, or poor mapping quality were excluded according to standard GATK hard-filtering criteria. Given the tumor-only sequencing design, common germline variants were filtered using population databases (including dbSNP and gnomAD), with variants showing a population allele frequency $\geq 1\%$ excluded unless previously reported as pathogenic in COSMIC or ClinVar.

To minimize FFPE-related artifacts, particularly cytosine deamination-associated C>T/G>A transitions, additional filtering steps were implemented, including exclusion of variants with strong strand bias or low supporting read counts. Only non-synonymous single-nucleotide variants, insertions/deletions, and clinically relevant copy number alterations were retained for analysis. These filtering and annotation steps were applied uniformly across all samples to ensure consistency and reproducibility.

A total of 135 patients with R/M HNSCC underwent NGS testing and constituted the “screened cohort”. Among them, 68 patients who met the eligibility criteria and received at least one cycle of anlotinib comprised the “treated cohort”. All analyses evaluating associations between genomic alterations and clinical outcomes (including response, PFS, and OS) were restricted to the treated cohort. The genomic landscape analysis describes the screened cohort, whereas outcome-associated analyses are based exclusively on the treated cohort.

Multiplex Immunofluorescence (mIF)

Multiplex immunofluorescence staining was carried out on serial FFPE sections (4 μm) using the Opal Polaris 7-Color Manual IHC Kit (Akoya Biosciences, Marlborough, MA, USA). The slides were deparaffinized, subjected to antigen retrieval in citrate buffer (pH 6.0), and incubated sequentially with primary antibodies against CD8 (clone C8/144B, Dako), CD68 (clone KP1, Abcam), CD163 (clone EPR19518, Abcam), PD-L1 (clone E1L3N, Cell Signaling Technology), and pan-Cytokeratin (AE1/AE3, Dako). Each primary antibody was detected with an HRP-conjugated secondary antibody, followed by tyramide signal amplification using distinct fluorophores. Nuclei were counterstained with DAPI. Five regions of interest (ROIs) per section were selected from viable tumor areas by two blinded pathologists. Whole-slide images were acquired using the Vectra Polaris Automated Quantitative Pathology Imaging System (Akoya Biosciences) and analyzed with inForm Advanced Image Analysis Software (v2.6). Positive and negative controls were included in each staining run. The densities of CD8⁺ T cells and macrophage subsets, their spatial proximity to tumor cells, and PD-L1 expression levels were quantified within tumor and stromal compartments. PD-L1 combined positive score (CPS) was calculated as:

$$\text{CPS} = \frac{\text{Number of PDL1 positive tumor cells, lymphocytes and macrophages}}{\text{Total viable tumor cells}}$$

where, a CPS ≥ 1 was defined as PD-L1 positive.

Efficacy and Safety Assessments

Tumor response was evaluated using computed tomography (CT) or magnetic resonance imaging (MRI) in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. Imaging was performed at baseline and subsequently at approximately 6- to 8-week intervals during treatment, consistent with routine clinical practice at our institution according to clinical circumstances, including patient symptoms and scheduling logistics. In the present cohort, the median imaging interval was 7.1 weeks (interquartile range, 6.3–8.4 weeks), and 82% of scans were completed within the intended 6–8 week window. All imaging studies were interpreted by board-certified radiologists specializing in head and neck oncology as part of routine clinical care, and treatment response was determined based on the radiology reports and imaging records documented in the medical charts.

ORR was defined as the proportion of patients achieving a complete response (CR) or partial response (PR), while DCR included patients with CR, PR, or stable disease (SD). PFS was measured from the initiation of anlotinib treatment to the date of documented disease progression or death, whichever occurred first. OS was defined as the time from treatment initiation to death from any cause.

Safety was assessed through the collection of adverse event data, which were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. Dose adjustments and treatment discontinuations due to AEs were documented to evaluate treatment tolerability.

Quality Control

All NGS and mIF experiments were performed in laboratories accredited under ISO 15189 standards. Negative and positive control tissues were included in each staining run. For NGS, sequencing quality metrics, including read depth, base quality, and mapping quality, were monitored to ensure data integrity, and variant calling was performed using standardized pipelines with predefined filtering criteria. Variant calls were validated in 10% of randomly selected cases by independent Sanger sequencing. Immunofluorescence quantification was performed in duplicate by two blinded pathologists, and inter-observer reproducibility exceeded 0.90 by the intraclass correlation coefficient.

Integration with Clinical Outcomes

Genomic alterations and immune-microenvironment parameters were correlated with clinical outcomes, including ORR, DCR, PFS, and OS. Associations between PIK3CA, PTEN, and EGFR status and treatment outcomes were assessed using Fisher's exact test for categorical variables and Cox proportional-hazards models for time-to-event data. Differences in immune cell infiltration and PD-L1 expression between responders and non-responders were compared using the Mann–Whitney *U*-test.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Clinical endpoints included ORR, DCR, PFS, and OS. ORR and DCR were summarized as proportions with corresponding 95% confidence intervals (CI) and compared between subgroups using the χ^2 or Fisher's exact test, as appropriate. PFS and OS were estimated using the Kaplan–Meier method, and survival differences between groups were assessed using the Log rank test. Hazard ratios (HR) and 95% CI were derived from univariable and multivariable Cox proportional-hazards regression models. Median follow-up duration was estimated using the reverse Kaplan–Meier method, which accounts for censoring and provides a more accurate estimate of potential follow-up time in survival analyses.

For biomarker analyses, PIK3CA, PTEN, EGFR, and FGF/FGFR pathway alterations identified by NGS were analyzed for their association with treatment outcomes. The relationships between genomic alterations and ORR were

assessed using Fisher's exact test, while associations with PFS and OS were evaluated using Cox regression models adjusted for age, tumor site, and PD-L1 CPS status.

Quantitative variables obtained from mIF, including densities of CD8⁺ T cells, CD68⁺ and CD163⁺ macrophages, and PD-L1 CPS, were compared between responders and non-responders using the Mann–Whitney *U*-test. Continuous variables with normal distribution were presented as mean $\pm$ standard deviation (SD) and compared using independent-samples *t*-tests, whereas non-normally distributed variables were presented as median (interquartile range, IQR). Correlations between immune infiltration, PD-L1 expression, and CBF values were evaluated using Spearman's rank correlation coefficient.

To account for multiple comparisons in exploratory biomarker analyses, the Benjamini–Hochberg false discovery rate (FDR) method was applied to two prespecified sets of comparisons: (i) associations between individual gene alterations and PFS derived from univariable Cox regression analyses, and (ii) comparisons of key immune microenvironment parameters (intratumoral CD8⁺ T-cell density and CD163⁺/CD8⁺ ratio) between responders and non-responders. For these analyses, both nominal *p*-values and FDR-adjusted *q*-values are reported. A *q*-value < 0.10 was considered potentially significant given the exploratory nature. All other subgroup analyses and secondary endpoints are presented with unadjusted *p*-values and should be interpreted as hypothesis-generating.

All other subgroup analyses and secondary endpoints are presented using unadjusted *p*-values and should be interpreted as exploratory or hypothesis-generating. All reported *P*-values were two-sided, and statistical significance was defined as $P < 0.05$ unless otherwise specified. Efficacy analyses were conducted in patients with available radiologic follow-up, while subgroup and biomarker analyses were performed in subsets with available PD-L1, NGS, or mIF data, as specified for each analysis.

Results

Patient Inclusion and Baseline Characteristics

A total of 68 patients with histologically confirmed recurrent R/M HNSCC who fulfilled the inclusion criteria were included. All patients were aged 18 years or older, had an ECOG performance status of 0–2, presented with at least one measurable lesion as defined by RECIST version 1.1, and had experienced disease progression after at least two prior lines of systemic therapy (ie., failure of second-line therapy). Patients with active brain metastases, uncontrolled hypertension, documented coagulation disorders, or prior exposure to anti-angiogenic TKIs were excluded.

The median age at the initiation of anlotinib treatment was 62 years (range, 42–78), and most patients were male (73.5%, 50/68) (Table 1). The most frequent primary tumor sites were the oropharynx (32.4%) and larynx (29.4%),

Table 1 Baseline Demographic and Clinical Characteristics of the Study Cohort

Characteristics	No. of Patients (%)	Characteristics	No. of Patients (%)
Median age (range), years	62 (42–78)	No. of metastatic sites	
Sex		1 site	25 (36.8)
Male	50 (73.5)	≥ 2 sites	43 (63.2)
Female	18 (26.5)	PD-L1 expression (CPS)	
Primary tumor site		CPS ≥ 1	32 (47.1)
Oropharynx	22 (32.4)	CPS < 1	29 (42.6)
Larynx	20 (29.4)	Unknown	7 (10.3)
Hypopharynx	16 (23.5)	No. of prior systemic therapy lines	
Oral cavity	10 (14.7)	2 lines	41 (60.3)
ECOG performance status		≥ 3 lines	27 (39.7)
0	18 (26.5)	Prior treatments	
1	35 (51.5)	Platinum-based chemotherapy	62 (91.2)
2	15 (22.0)	PD-1/PD-L1 inhibitor	58 (85.3)
Karnofsky performance status		Cetuximab	27 (39.7)
61–70	22 (32.3)	Radiotherapy	65 (95.6)
71–80	35 (51.5)		
81–90	11 (16.2)		

which is consistent with global epidemiological distributions of HNSCC. A substantial proportion of patients (63.2%) exhibited multi-organ metastases at baseline, reflecting the advanced disease status of the cohort. Regarding prior therapies, 91.2% of patients had received platinum-based chemotherapy, 85.3% had been treated with immune check-point inhibitors, and 39.7% had been received anti-EGFR targeted therapy. These findings indicate that population consisted of patients with failure of second-line systemic therapy and subsequent disease progression in a heavily pretreated post-second-line failure setting.

Efficacy Outcomes

Among the 68 treated patients, the best overall tumor responses were as follows: 14 patients (20.5%) achieved PR, 39 (57.4%) had SD, and 15 (22.1%) experienced PD. Efficacy analysis in patients with post-second-line failure treated with anlotinib revealed an ORR of 22.1%, and DCR of 74.6% (Table 2). Maximum tumor shrinkage was observed around week 9 following treatment initiation. Importantly, radiological responses were documented within the first three treatment cycles in 86.7% of patients, indicating an early onset of anti-tumor activity with anlotinib.

Subgroup analysis of patients with PD-L1-negative tumors (CPS <1), generally considered less likely to respond to immunotherapy, revealed an ORR of 17.2% and a DCR of 70.9% (Table 2). These findings suggest that the clinical activity of anlotinib may be independent of PD-L1 expression, indicating its potential in patients with low expected responsiveness to immunotherapy.

Survival Outcomes

As of the data cutoff in December 2024, the median follow-up duration for the overall cohort was 15.3 months (interquartile range [IQR], 10.2–21.8; range, 2.8–30.1), estimated using the reverse Kaplan–Meier method. At the time of analysis, 45 of 68 patients (66.2%) had experienced disease progression, and 38 patients (55.9%) had died, while the remaining patients were censored at the date of last follow-up. One patient was censored before the first scheduled imaging assessment due to withdrawal of consent. For those alive at last contact, the median follow-up duration was 16.8 months (IQR, 11.5–23.2), indicating adequate observation time for outcome assessment.

The median progression-free survival (mPFS) was 6.3 months (95% CI, 4.2–7.5) (Figure 1), which compares favorably with historical estimates of 2.0–3.5 months for patients with post-second-line failure receiving monotherapy in R/M HNSCC. The 6-month PFS rate was 42.6%, indicating that a substantial proportion of patients achieved disease stabilization beyond six months.

The median overall survival (mOS) was 8.4 months (95% CI, 7.8–10.6) (Figure 2), exceeding the approximately 6 months typically reported in patients receiving best supportive care alone. The 12-month OS rate was 34.8%, further supporting the survival benefit of anlotinib in this post-second-line failure population.

Table 2 Subgroup Analysis of Efficacy Outcomes with Anlotinib as Third-Line or Later Treatment (n = 48*)

Efficacy Parameters	Overall Cohort	PD-L1 CPS ≥1 (n = 27)	PD-L1 CPS <1 (n = 19)
Objective response rate (ORR), %	22.1	24.6	17.2
Disease control rate (DCR), %	74.6	79.3	70.9
Median progression-free survival (PFS), months	4.8	5.1	4.5
6-month PFS rate, %	42.6	46.9	41.4
Median overall survival (OS), months	9.2	10.1	8.7
12-month OS rate, %	34.8	37.5	31.0

Notes: *PD-L1 CPS status was available in 61 of the 68 enrolled patients. Among them, 48 patients had complete imaging follow-up and survival data and were included in the PD-L1 efficacy analysis (CPS ≥1, n=27; CPS <1, n=19; two patients were censored before first radiologic assessment). The remaining 13 patients were excluded from subgroup efficacy evaluation due to insufficient data for objective response or time-to-event endpoints (e.g., early loss to follow-up, imaging not performed, or death before first response assessment). Thus, efficacy outcomes were calculated only in treated patients with standardized RECIST-based follow-up.

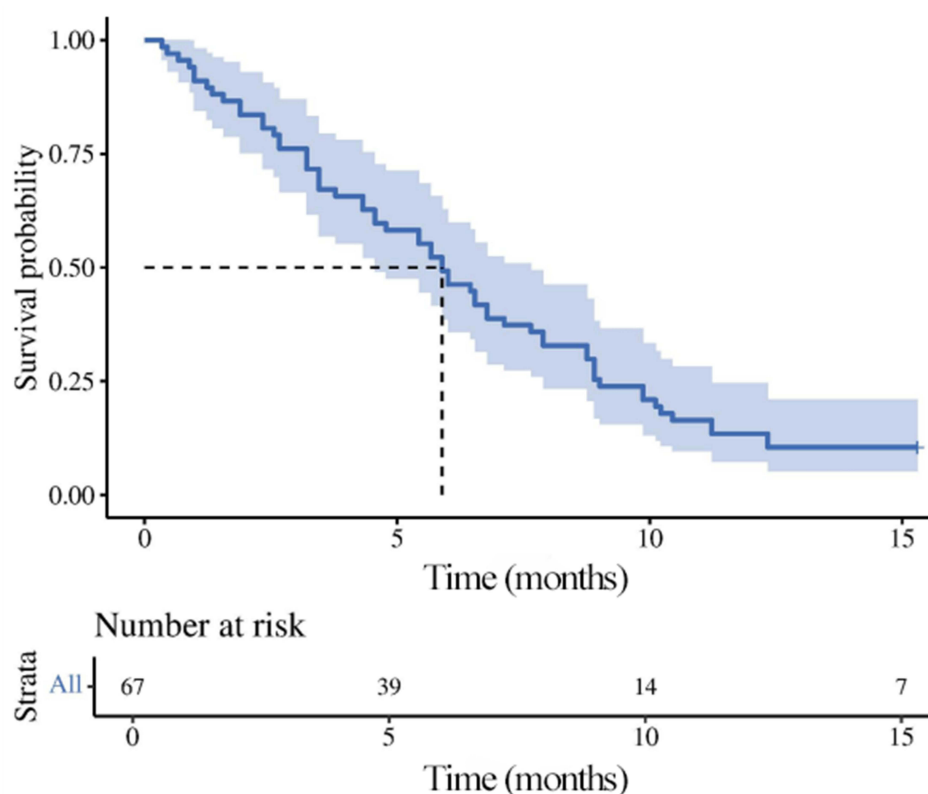


Figure 1 Progression-Free Survival of Patients Treated with Anlotinib. Kaplan–Meier curve of the progression-free survival (PFS) for all enrolled patients with recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC) treated with anlotinib ($n = 68$). The median PFS was 6.3 months (95% CI, 4.2–7.5). The 6-month PFS rate was 42.6%, indicating durable disease control in a substantial subset of patients who had progressed after both platinum-based chemotherapy and immune checkpoint inhibitors. Censoring events are indicated by tick marks. The number at risk at time 0 is 68. One patient was censored before the first scheduled imaging assessment (week 6) due to withdrawal of consent and was therefore not included in subsequent risk sets.

For PFS analysis, 23 patients (33.8%) were censored. The reasons included ongoing treatment without documented progression at the data cutoff ($n = 14$, 20.6%), loss to follow-up before progression ($n = 5$, 7.4%), treatment discontinuation due to adverse events without progression ($n = 3$, 4.4%), and withdrawal of consent ($n = 1$, 1.5%). For OS analysis, 30 patients (44.1%) were censored, all of whom were alive at the last follow-up. No patients were lost to follow-up for vital status, as survival status was confirmed through medical records or telephone contact within 3 months before the data cutoff. Detailed patient-level censoring information is provided in [Supplementary Table S1](#).

Subgroup Analysis

Subgroup analyses based on baseline clinical variables demonstrated consistent treatment efficacy across multiple patient strata ([Table 3](#)). Among different primary tumor locations, patients with oropharyngeal carcinoma achieved the longest mPFS (7.6 months), followed by those with laryngeal (6.8 months) and hypopharyngeal (4.1 months) carcinomas. Performance status was a significant predictor of clinical outcome: patients with ECOG PS 0–1 had a significantly longer mPFS compared to those with PS 2 (6.8 vs. 5.1 months; $P = 0.002$), emphasizing the prognostic value of functional status.

Importantly, patients who had previously demonstrated primary resistance to immunotherapy still achieved clinical benefit from anlotinib, with an mPFS of 6.2 months. No statistically significant differences in efficacy outcomes were observed between patients with and without prior immunotherapy exposure, suggesting no evident interaction between prior treatment type and treatment efficacy in this post-second-line failure setting. Furthermore, patients with cervical lymph node metastases ($n = 31$) exhibited higher response rates, with an ORR of 29.0% and a DCR of 83.9%. This enhanced efficacy may be partially attributed to improved local drug delivery, potentially facilitated by anlotinib-induced vascular remodeling and enhanced tumor perfusion.

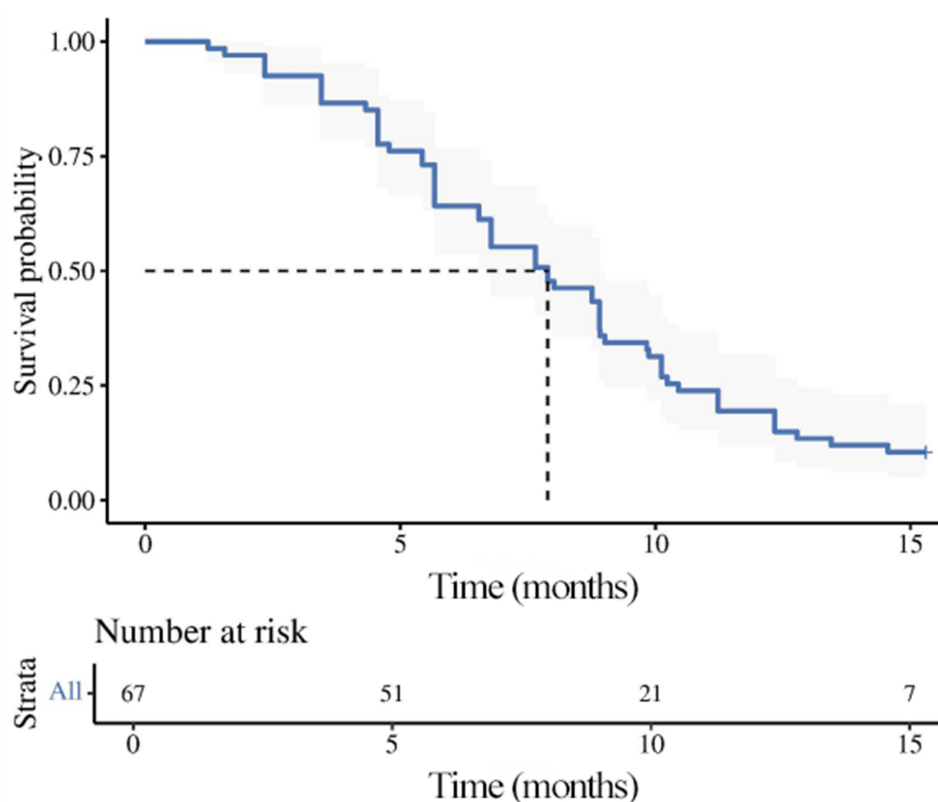


Figure 2 Overall Survival of Patients Treated with Anlotinib. The median OS was 8.4 months (95% CI, 7.8–10.6), and the 12-month OS rate was 34.8%, exceeding historical expectations for third-line or later therapy in R/M HNSCC. Censoring events are indicated by tick marks. The number at risk at time 0 is 68. One patient was censored before the first scheduled imaging assessment (week 6) due to withdrawal of consent and was therefore not included in subsequent risk sets.

Safety Profile

Treatment-related AEs were generally manageable across the cohort (Table 4). Grade ≥ 3 AEs were observed in 25.0% of patients (17/68), with hypertension (11.8%), hand-foot syndrome (7.4%), and fatigue (5.9%) being the most frequently reported high-grade toxicities. One patient discontinued treatment due to grade 3 epistaxis; however, no treatment-related

Table 3 Subgroup Analyses of Progression-Free Survival (PFS), Objective Response Rate (ORR), and Disease Control Rate (DCR)

Subgroup	Patients, n	Median PFS (Months)	ORR (%)	DCR (%)	Statistical Significance
Primary tumor site					
Oropharyngeal cancer	22	7.6	35.2	85.7	—
Laryngeal cancer	20	6.8	28.6	80.0	—
Hypopharyngeal cancer	16	4.1	15.8	68.8	$p < 0.001$ (vs. oropharyngeal cancer)
ECOG performance status					
0–1	53	6.8	25.4	82.5	$p = 0.002$ (vs. PS 2)
2	15	5.1	13.3	66.7	—
Prior immunotherapy (IO) response					
Primary resistance	41	6.2	20.5	75.6	—
Metastatic pattern					
Cervical lymph node metastasis	31	—	29.0	83.9	$p = 0.03$ (ORR vs. non-cervical metastasis)
Non-cervical metastasis	37	—	16.2	73.0	—

Note: Genomic subgroup definitions were derived from NGS results obtained in the screened cohort (overall cohort) but analyzed only in treated patients (n=68) with available outcome data.

Table 4 Treatment-Related Adverse Events

Adverse events	All grades, n (%)	Grade ≥ 3 , n (%)
Hypertension	38 (55.9)	8 (11.8)
Hand-foot syndrome	30 (44.1)	5 (7.4)
Fatigue	28 (41.2)	4 (5.9)
Decreased appetite	25 (36.8)	3 (4.4)
Proteinuria	18 (26.5)	2 (2.9)
Thyroid dysfunction	12 (17.6)	1 (1.5)
Hemorrhagic events	8 (11.8)	2 (2.9)

deaths occurred during the study period. Oral mucositis was reported in 14.7% of patients, a slightly higher incidence than that observed in anlotinib trials for lung cancer. This may be attributable to the prior administration of radiotherapy and the heightened anatomical sensitivity of the head and neck mucosal tissues. Notably, only one case of grade 3 mucositis was documented.

Overall, 6 of the 68 patients (8.8%) discontinued anlotinib due to AEs, which is lower than that reported for other TKIs in similar advanced refractory settings, which typically exceeds 15%, and supports the overall tolerability of anlotinib in post-second-line treatment of R/M HNSCC.

Dose Adjustment and Treatment Compliance

Dose modifications were frequently required and reflected routine clinical practice aimed at balancing efficacy and tolerability. Among the 42 patients who initiated anlotinib at the standard dose of 12 mg/day, 23 (54.8%) required a dose reduction to 10 mg, and an additional 10 patients (23.8%) were further reduced to 8 mg due to treatment-related AEs. Of the 26 patients who began therapy at a reduced starting dose of 10 mg, typically due to advanced age or poor performance status, 17 (65.4%) required subsequent reduction to 8 mg. Despite the need for dose adjustments, treatment compliance remained high. The mean relative dose intensity was 78.5%, indicating that most patients were able to maintain sufficient drug exposure throughout the treatment course. This supports the feasibility of individualized dose optimization strategies to enhance tolerability without compromising therapeutic efficacy in post-second-line failure patients.

Genomic Landscape of the Anlotinib-Screened Cohort

NGS was performed on archived tumor tissues from the 135 patients, defined as the screened cohort, to characterize genomic alterations in cancer-related pathways and among them, 68 patients received anlotinib and were classified as the treated cohort (Figure 3). Among the screened patients (including the 68 treated cases), recurrent genomic alterations were observed in TP53 (45.2%), CDKN2A (13.3%), PIK3CA (13.3%), TERT (13.3%), LRP1B (12.6%), and FAT1 (11.1%). PTEN alterations comprised both mutations and copy number loss. Alterations involving the FGF/FGFR pathway were infrequent (~5%) and included gene amplification and activating mutations. The additional mutations identified were in NOTCH1, ARID1A, SMARCA4, CCND1 and other cancer-related genes, highlighting the molecular heterogeneity of this cohort.

Correlation of Genomic Alterations with Therapeutic Efficacy

To explore genomic predictors of outcome, we compared progression-free survival according to PI3K pathway status based on the 68 patients in the treated cohort with available clinical outcome data. The Kaplan–Meier curves showed no significant difference in PFS between patients with PI3K pathway alterations and those with wild-type tumors (log-rank $p = 0.40$), and the hazard ratio favored the wild-type cohort (Figure 4A). Consistently, tumors with PI3K pathway activation (defined as PIK3CA mutation or PTEN loss) did not demonstrate a survival advantage (Figure 4B).

In univariable Cox regression analyses of individual gene alterations (Figure 4B), alterations in PIK3CA, PTEN, MTOR, and several related genes were not associated with improved PFS, and in some cases showed a trend toward shorter survival. In univariable Cox regression analyses of individual gene alterations (Figure 4B), none of the evaluated

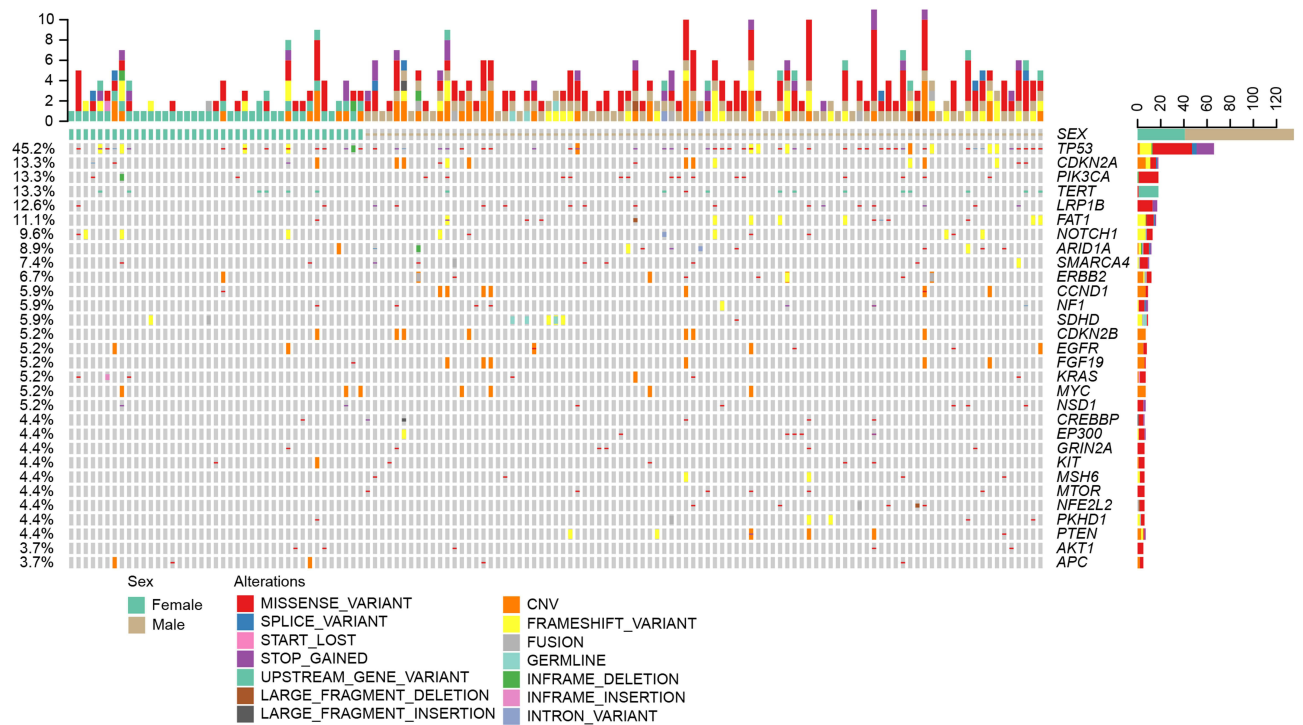


Figure 3 Genomic Landscape of Screened Head and Neck Squamous Cell Carcinoma (HNSCC) Patients (n = 135). Targeted next-generation sequencing (NGS) was performed on tumor samples from 135 patients with recurrent or metastatic head and neck squamous cell carcinoma, defined as the screened cohort. This cohort includes the subset of 68 patients who subsequently received anlotinib (treated cohort). The most frequently altered genes were TP53 (45.2%), CDKN2A (13.3%), PIK3CA (13.3%), TERT (13.3%), LRP1B (12.6%), and FAT1 (11.1%). Additional alterations were identified in NOTCH1, ARID1A, SMARCA4, CCND1, and PTEN. Alterations in the FGF/FGFR pathway were infrequent (~5%) and included gene amplification and activating mutations; no gene fusions were detected. PTEN loss refers specifically to copy-number deletion. Genes are ranked by alteration frequency, and mutation types (missense, frameshift, truncation, and copy-number gain/loss) are color-coded. This figure summarizes the mutational spectrum of the screened cohort; analyses correlating genomic alterations with clinical outcomes were conducted separately in the treated cohort (n = 68). Genes are ordered by mutation frequency, and mutation types are grouped to improve readability.

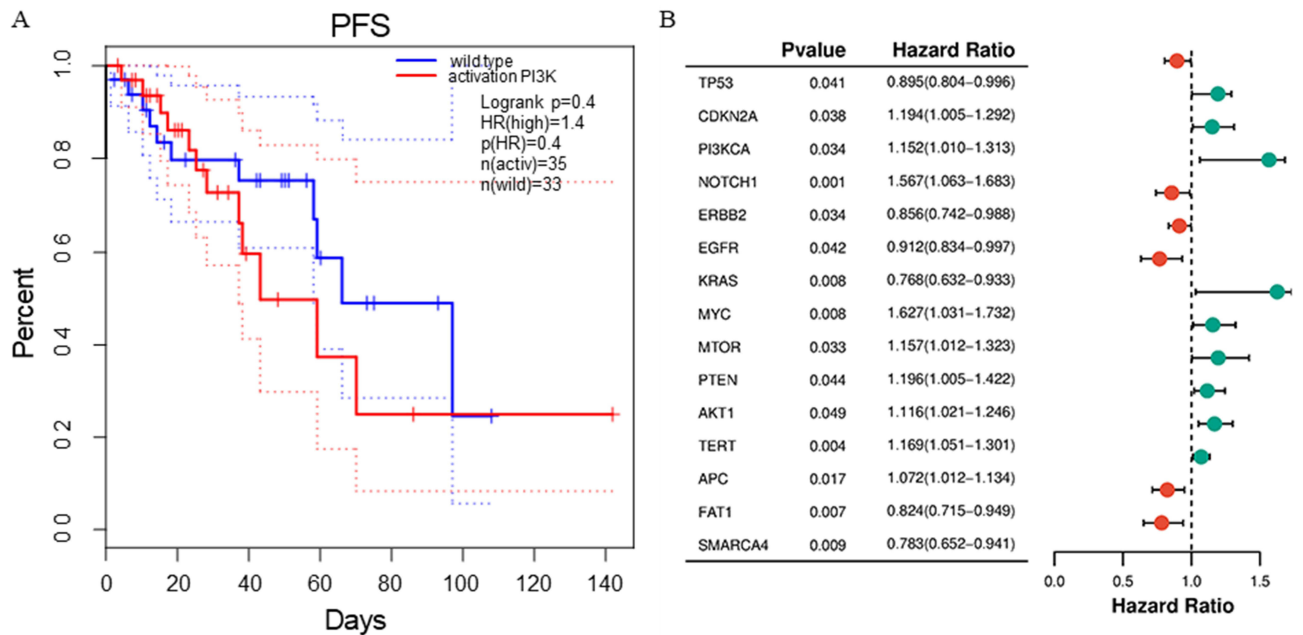


Figure 4 Correlation of Key Genomic Alterations with Anlotinib Efficacy. Analyses were restricted to patients who received anlotinib and had evaluable outcome data (n=68). (A) Kaplan–Meier curves comparing progression-free survival (PFS) between patients with PI3K pathway alterations (defined as PIK3CA mutation or PTEN loss) and those without such alterations; no significant difference was observed (log-rank p = 0.40). (B) Forest plot of hazard ratios for PFS derived from univariable Cox regression analyses of selected gene alterations. Nominal p-values are shown; after Benjamini-Hochberg FDR correction (q<0.10), no alteration remained significant. HRs are plotted on a logarithmic scale; error bars represent 95% confidence intervals.

genomic alterations showed a significant association with PFS after FDR correction. For example, PIK3CA (HR = 1.4, 95% CI 0.9–2.1; $p = 0.12$, $q = 0.24$), PTEN (HR = 1.3, 95% CI 0.8–2.0; $p = 0.21$, $q = 0.31$), and FGF/FGFR (HR = 1.5, 95% CI 0.9–2.4; $p = 0.08$, $q = 0.19$) were not significantly associated with PFS after FDR correction. Overall, these findings indicate that the assessed genomic alterations were not associated with clinical benefit from anlotinib in this cohort.

Immune Microenvironment Features Assessed by mIF

mIF analysis was conducted to evaluate tumor-immune interactions and PD-L1 expression in patients from the treated cohort to evaluate the immune microenvironment and its association with treatment response. The densities of CD8⁺ T cells and CD163⁺ macrophages within the tumor nest and stromal compartments were quantified using inForm software (Akoya Biosciences), and differences between responders and non-responders were compared using the Mann–Whitney *U*-test. The results indicated that responders (PR + SD ≥ 6 months) had significantly higher intratumoral CD8⁺ T-cell density than non-responders (median 386 vs. 112 cells/mm², $p = 0.008$), with the CD163⁺/CD8⁺ ratio being significantly lower in responders (median 1.2 vs. 3.4, $p = 0.01$) (Figure 5). In patients with tumors showing PD-L1 CPS ≥ 1 , this pattern was consistent with a more inflamed immune phenotype, characterized by increased CD8⁺ T-cell infiltration and a relatively lower burden of CD163⁺ macrophages (Figure 5). These cases were more likely to achieve radiologic response and sustained disease control. In contrast, tumors with sparse CD8⁺ T-cell infiltration or a predominance of CD163⁺ macrophages showed a more immunosuppressive profile and were generally associated with early progression. Spatially, responding tumors displayed dense CD8⁺ cells within tumor nests, whereas non-responding tumors showed only scattered CD8⁺ cells, often restricted to stromal or perivascular regions. These differences remained significant after Benjamini–Hochberg FDR correction within the prespecified immune biomarker analyses ($q < 0.10$). However, given the limited sample size and exploratory nature of these analyses, these findings should be interpreted with caution.

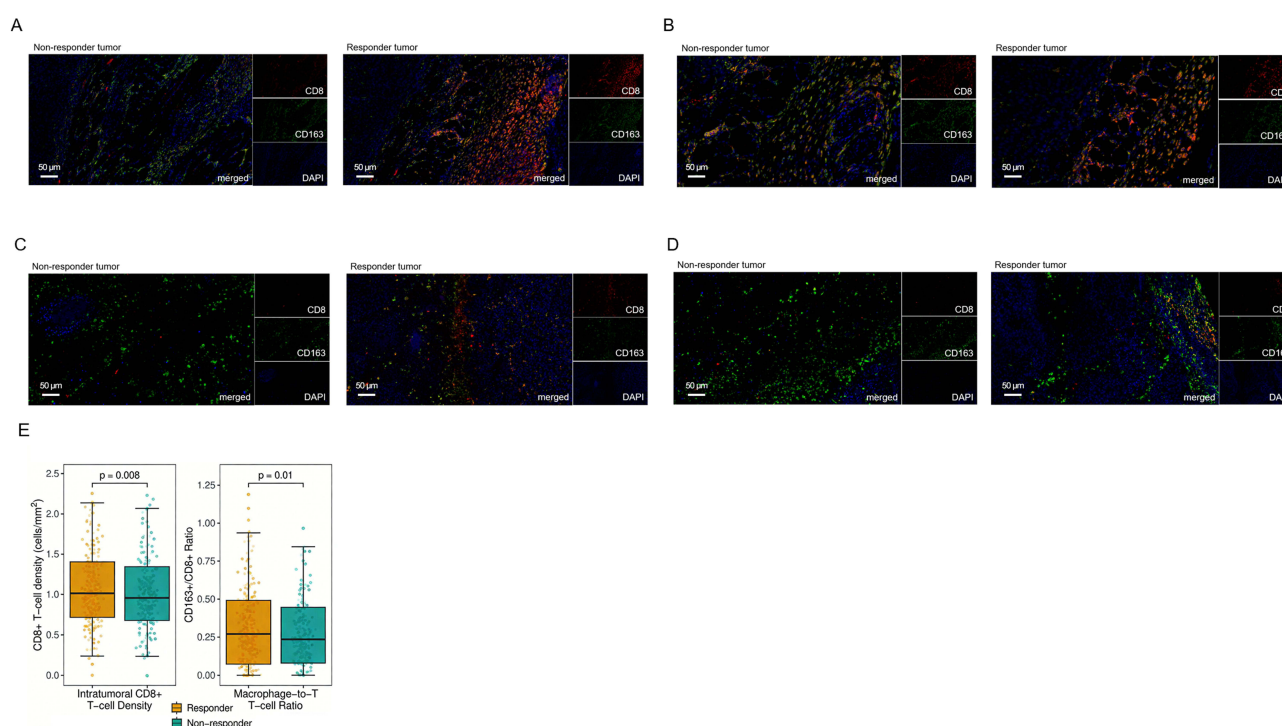


Figure 5 Multiplex Immunofluorescence Images of the Tumor Immune Microenvironment in Four Representative Cases of Head and Neck Squamous Cell Carcinoma. Representative images of responders and non-responders with tumors from the (A) oral cavity, (B) oropharynx, (C) hypopharynx, and (D) larynx. In responding tumors, CD8⁺ T cells (red) can be observed within tumor nests and are more abundant overall, while CD163⁺ M2 macrophages (green) are reduced. Non-responding tumors show the opposite pattern, with few CD8⁺ cells and a predominance of CD163⁺ cells, often limited to stromal or perivascular areas. The nuclei were counterstained with DAPI (blue). The merged images demonstrate the spatial relationship between immune cells and tumor regions. Scale bar = 50 μ m. (E) Quantitative comparisons of CD8⁺ T-cell density and CD163⁺/CD8⁺ ratio between responders and non-responders are shown, with statistical significance assessed using the Mann–Whitney *U*-test.

Discussion

This retrospective analysis provides real-world evidence supporting the efficacy and tolerability of anlotinib in patients with R/M HNSCC who had progressed following both platinum-based chemotherapy and immune checkpoint inhibition. In this cohort of patients with post-second-line failure (defined as progression after at least two prior systemic regimens), anlotinib yielded an ORR of 20.5% and a DCR of 78.0%, with an mPFS of 6.3 months and mOS of 8.4 months. These findings are particularly notable given the limited treatment options and poor prognosis typically associated with third-line or post-second-line treatment setting in R/M HNSCC.

The clinical efficacy of anlotinib in our study compares favorably with other post-second-line treatment options for R/M HNSCC. Historical data suggest that conventional cytotoxic agents in the post-second-line treatment setting typically yield response rates of less than 10% and PFS of approximately 2.0–3.5 months.²¹ A recent analysis by Argiris et al reported a median OS of just 4–5 months for patients with R/M HNSCC receiving treatment in the post-second-line setting.²² In this context, our findings represent a significant improvement in outcomes and underscore the potential clinical utility of anlotinib in this patient population. The observed efficacy of anlotinib may be attributed to its unique molecular profile and mechanism of action. By targeting multiple angiogenic and oncogenic pathways simultaneously, anlotinib may overcome compensatory resistance mechanisms that limit the effectiveness of more selective TKIs.²³ The inhibition of VEGFR, FGFR and PDGFR signaling not only disrupts tumor vasculature but also modulates the immunosuppressive tumor microenvironment, potentially enhancing anti-tumor immune responses,²⁴ and this is particularly relevant in HNSCC, where dysregulated angiogenesis and immune evasion are key contributors to disease progression and treatment resistance.

Importantly, our findings revealed meaningful clinical benefit even in patients with traditionally poor prognostic factors. Subgroup analysis demonstrated responses across various anatomical subsites, including hypopharyngeal and laryngeal primaries, which typically carry worse outcomes.²⁵ Furthermore, the efficacy observed in patients with PD-L1 CPS <1 (“cold tumors”) and those with prior resistance to immunotherapy suggests that anlotinib may address important unmet needs in these difficult-to-treat subpopulations. The potential synergy between anti-angiogenic therapy and immunotherapy has gained increasing attention in recent years. In this regard, Jain et al proposed that judicious use of anti-angiogenic agents could normalize tumor vasculature, enhance T-cell infiltration, and overcome resistance to immune checkpoint blockade.²⁶ Our observation that anlotinib demonstrated activity in immunotherapy-refractory patients aligns with emerging evidence from other tumor types. Furthermore, Luo et al recently reported that combining anlotinib with PD-1 inhibitors could reverse acquired resistance to immunotherapy in NSCLC through modulation of the tumor microenvironment and upregulation of chemokines such as CCL5.²⁷ Similar mechanisms may underlie the efficacy of anlotinib in R/M HNSCC, although further translational studies are needed to elucidate the precise molecular interactions. In addition, although the cohort included patients with heterogeneous prior treatments, no significant differences were observed across these subgroups, supporting the interpretability of pooled efficacy outcomes in this real-world setting.

The safety profile of anlotinib in our cohort was consistent with previous reports in other malignancies. The most common adverse events, namely hypertension, hand-foot syndrome, and fatigue, were generally manageable with dose modifications or supportive care. The incidence of grade ≥ 3 adverse events (25.0%) was lower than that reported with other multi-targeted TKIs in similar settings.²⁸ Notably, the treatment discontinuation rate of 8.8% due to toxicity compares favorably with other TKIs in HNSCC, which typically exceed 15%,²⁹ suggesting that anlotinib may offer a more tolerable treatment option for patients with post-second-line treatment failure.

In this cohort, alterations in PIK3CA or PTEN did not identify a group with better PFS. When we compared patients with and without these alterations, the survival curves were essentially overlapping, and the hazard ratios did not favor the mutated group, suggesting no clear clinical benefit with anlotinib in patients carrying PI3K pathway changes. Although laboratory studies have suggested that PI3K activation may increase reliance on angiogenic signaling, our clinical data did not demonstrate this effect. Alterations involving the FGF/FGFR pathway were seen only in a small number of cases, indicating a need for confirmation in larger-scale studies.

The association of PD-L1 CPS ≥ 1 and high CD8⁺ T-cell infiltration with higher ORR and longer PFS in our cohort is compatible with the vascular-immune normalization paradigm. For instance, anti-angiogenic agents can transiently normalize

abnormal tumor vessels, reduce hypoxia, and improve lymphocyte trafficking, thereby enhancing cytotoxic T-cell function and potentially re-sensitizing tumors to immune attack, even in previously immunotherapy-refractory disease. Recent reviews synthesize clinical and preclinical evidence for this bidirectional crosstalk, and mechanistic work demonstrates that VEGF/VEGFR inhibition can augment anti-PD-(L)1 efficacy by fostering T-cell infiltration and IFN- γ -driven PD-L1 upregulation within tumors.^{30,31} Within HNSCC, specifically, where immune evasion and aberrant angiogenesis coexist, these effects may be particularly relevant. Our finding that clinical benefit was still observed in a subset with prior checkpoint inhibitor resistance aligns with this framework and merits prospective validation with predefined immune-vascular endpoints.

Real-world and early clinical experiences combining or sequencing anlotinib with PD-1 blockade in R/M HNSCC report encouraging activity with manageable toxicity, supporting our observation that anti-angiogenic therapy can provide meaningful benefit after checkpoint inhibitors or EGFR-directed therapy.^{32,33} Beyond HNSCC, multi-tumor evidence continues to reinforce the anti-angiogenesis and immunotherapy strategy, lending external validity to our integrated biomarker–efficacy signals and providing a rationale for future combination trials in biomarker-enriched HNSCC subsets.³⁴

Although EGFR alterations were infrequent in our cohort, their modest association with benefit is biologically coherent: EGFR activation upregulates VEGF and other angiogenic mediators in HNSCC, thereby coupling mitogenic and angiogenic programs. This linkage provides a mechanistic basis for the observed sensitivity to a multikinase anti-angiogenic agent, suggesting that EGFR-altered tumors may still be exploitable through angiogenesis inhibition even when classic EGFR-targeted strategies have failed.^{35,36}

Despite these encouraging findings, several limitations should be acknowledged. First, the retrospective design might have introduced selection bias and residual confounding. Second, the absence of a control group limits direct comparison with other post-second-line treatment approaches. Third, not all treated patients had complete NGS and mIF data, which reduced the sample size available for translational analyses. Fourth, our DNA-based NGS approach does not capture dynamic transcriptional changes or the functional cellular impact of anlotinib. Transcriptomic methods (eg., RNA-seq) are better suited for this purpose, and their absence limits mechanistic interpretation. Future studies integrating RNA-seq are warranted. In addition, although NGS and mIF were added to provide molecular and immune context, these analyses are mainly exploratory and not intended to establish a validated biomarker-guided model for treatment selection. The observed associations should therefore be interpreted with caution. Moreover, genomic profiling was performed in the screened cohort ($n = 135$), whereas outcome-correlative analyses were restricted to the treated cohort ($n = 68$), which may also limit the strength of translational inference. Lastly, given the limited sample size of the treated cohort and the exploratory nature of multi-biomarker analyses, these analyses may not be powered for definitive inference. All biomarker-related findings should be therefore interpreted as hypothesis-generating. Overall, due to the marked molecular heterogeneity of HNSCC, larger prospective studies with predefined biomarker hypotheses and more complete paired profiling will be needed to determine whether these signals can be translated into clinically useful patient-selection strategies.

In conclusion, anlotinib demonstrated encouraging antitumor activity with a manageable safety profile in heavily pretreated patients with R/M HNSCC with post-second-line treatment failure. Clinical benefit was also observed in patients with prior immunotherapy resistance and in those with PD-L1–negative tumors, supporting its potential role in this difficult-to-treat setting. Exploratory translational analyses suggested that tumors with PD-L1 CPS ≥ 1 and higher CD8⁺ T-cell infiltration were more likely to exhibit favorable outcomes, consistent with an inflamed immune microenvironment, whereas PI3K pathway alterations were not associated with prolonged PFS in this cohort. Taken together, these findings support the potential value of anlotinib as a post-second-line treatment option and provide hypothesis-generating insight into the integration of genomic and immune biomarkers for patient stratification. Further prospective studies are required to validate these observations.

Abbreviations

AE, adverse event; CI, confidence interval; CPS, combined positive score; CR, complete response; CT, computed tomography; CTCAE, Common Terminology Criteria for Adverse Events; DCR, disease control rate; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; HNSCC, head and neck squamous cell carcinoma; ICI, immune checkpoint inhibitor; MRI, magnetic resonance imaging; mOS, median overall survival; mPFS, median progression-free survival; NSCLC, non-small

cell lung cancer; ORR, objective response rate; OS, overall survival; PD, progressive disease; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PDGFR, platelet-derived growth factor receptor; PFS, progression-free survival; PR, partial response; PS, performance status; RECIST, Response Evaluation Criteria in Solid Tumors; R/M, recurrent or metastatic; SD, stable disease; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Data Sharing Statement

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

Ethics Approval

Ethical approval was given by Tianjin Medical University's ethics committee (Ethics Approval Number: EK2024017).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare that they have no conflicts of interest.

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