

Correlation of p16 Protein Status With MicroRNA Expression in Squamous Cell Carcinoma of the Mobile Tongue: A Pilot Study

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Abstract

Background

The role of p16 as a surrogate marker for human papillomavirus (HPV) infection in pure mobile-tongue squamous cell carcinoma (TSCC) remains controversial, and potential associations between p16 status and microRNA (miRNA) expression profiles are largely unexplored.

Objective

The objective of this study was to perform a pilot, hypothesis-generating correlative analysis of p16 protein expression (used here strictly as a surrogate marker, without direct HPV testing) and pre-existing miRNA data in an archival cohort of mobile-TSCCs, with the aim of nominating candidate miRNAs for future validation rather than identifying a definitive miRNA signature.

Methods

Paired tumor and adjacent non-neoplastic specimens from 12 consecutive patients with primary mobile-TSCC who underwent surgical resection between 2010 and 2011 were previously profiled for mature miRNA expression. After exclusion of one sample for failure of the original quality-control criterion and one further case with incomplete p16 or miRNA data, a final matched cohort of 10 patients was analyzed. p16 immunohistochemistry was performed on formalin-fixed paraffin-embedded tumor sections; positivity was defined as moderate-to-intense nuclear and cytoplasmic staining in $\geq 70\%$ of neoplastic cells. Associations between tumor miRNA cycle-threshold values (84 mature human miRNAs) and p16 status, as well as other clinicopathological variables, were examined with non-parametric tests and Benjamini-Hochberg false-discovery-rate (FDR) correction.

Results

Two of the 10 tumors (20%) were classified as p16-positive. Twelve miRNAs exhibited nominal differences between p16-positive and p16-negative tumors (uncorrected Mann-Whitney $p = 0.044$ for all 12); none remained statistically significant after FDR correction (all $FDR \geq 0.31$). The most pronounced numerical elevations in the p16-positive subset were observed for miR-150-5p (approximate fold-change 9.6), miR-206 (6.9), miR-155-5p (4.9), miR-146a-5p (4.0) and miR-29b-3p (3.8). No robust associations were identified with patient outcome, sex, T-stage, histological differentiation, tumor side, hospital of origin, or age. The study was not powered to detect moderate effect sizes and all analyses are exploratory.

Conclusions

In this small pilot cohort, p16 status showed only nominal, non-FDR-significant differences in miRNA expression. The numerical elevation of the established oncomiR miR-155-5p in the two p16-positive tumors is hypothesis-generating but requires confirmation in larger multi-institutional series that incorporate both rigorous p16 assessment and direct HPV molecular testing.

Categories: Otolaryngology, Pathology, Oncology

Keywords: carcinoma, cyclin-dependent kinase inhibitor p16, micrornas, papillomavirus infections, squamous cell, tongue neoplasms

Introduction

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of malignancies arising in the oral cavity and remains a major cause of cancer-related morbidity and mortality worldwide [1,2]. Within the oral cavity,

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squamous cell carcinoma of the mobile (oral) tongue (TSCC) is the most frequent and biologically aggressive subtype, characterized by early local invasion, frequent regional lymph-node metastasis, and relatively poor five-year survival [3]. Despite advances in surgical technique, radiotherapy, and systemic therapy, clinical outcomes have improved only modestly over recent decades, underscoring the need for a deeper understanding of the molecular drivers of TSCC and for reliable biomarkers that can refine risk stratification and guide targeted interventions [4].

MicroRNAs (miRNAs) are short, endogenous non-coding RNAs that post-transcriptionally repress target mRNAs and thereby regulate virtually every cellular process relevant to carcinogenesis, including proliferation, apoptosis, differentiation, invasion, and immune modulation [5-7]. Multiple miRNAs are recurrently dysregulated in OSCC/TSCC [8-10]. Among them, miR-21, miR-155, miR-146a, and members of the miR-200 family have been repeatedly implicated as oncogenic or tumor-suppressive drivers [11-13]. In particular, miR-155-5p has emerged as a multifunctional oncomiR whose overexpression is associated with advanced stage, lymph-node metastasis, therapy resistance, and unfavorable prognosis in OSCC [14,15]. Experimental work has further shown that miR-155 participates in feedback loops that promote tumorigenesis in tongue cancer cells [16,17].

Human papillomavirus (HPV) infection, principally high-risk type 16, is a well-established etiological factor in oropharyngeal squamous cell carcinoma, where it is tightly linked to p16INK4a overexpression and a distinctly favorable prognosis [18,19]. In contrast, the role of HPV (and its surrogate marker p16) in pure oral-cavity and especially mobile-TSCC remains controversial and appears far less frequent [20]. Nevertheless, a subset of oral-tongue tumors does express p16, raising the possibility that HPV-driven molecular programs, including specific miRNA signatures, may still operate in a minority of cases and influence biological behavior [16].

In 2019, we reported a systematic miRNA profiling study of paired tumor and adjacent normal tissue from 12 patients with mobile-TSCC treated at 2 tertiary centers in Thessaloniki [21]. That work identified a panel of markedly up-regulated (miR-196a, miR-142, miR-96, miR-21) and down-regulated (miR-133b, miR-206, miR-1) miRNAs and suggested possible links to prognosis. The present study extends those observations by examining the same archival cohort for p16 protein expression and by correlating the pre-existing miRNA data with p16 status and other clinicopathological variables.

Our primary aim was to explore, in a strictly hypothesis-generating manner, whether p16-positive mobile-TSCCs (defined by immunohistochemistry alone, without confirmatory HPV testing) exhibit numerical differences in miRNA expression relative to p16-negative tumors, with particular attention to miR-155-5p and other candidate oncomiRs. The goal was to nominate candidates for future multi-institutional studies that combine rigorous p16 assessment with direct HPV molecular testing, rather than to identify or validate a definitive miRNA signature.

Materials And Methods

Study design and patient cohort

This study is a continuation of a previously published investigation on microRNA expression profiles in mobile-TSCC [21]. The original cohort consisted of 12 consecutive patients with primary TSCC of the anterior (body) of the tongue who underwent surgical resection at the Departments of Oral and Maxillofacial Surgery of the “Theagenion” Cancer Hospital and the “G. Papanikolaou” General Hospital of Thessaloniki between April 2010 and May 2011. None of the patients had received radiotherapy, chemotherapy, or any other antineoplastic treatment prior to surgery. Written informed consent for tissue donation for research purposes had been obtained from all patients, and the original protocol had been approved by the Ethics Committee of Aristotle University of Thessaloniki.

In the present extension of that work, the same archival cases were re-evaluated for p16 protein expression by immunohistochemistry, and the existing microRNA expression data were correlated with p16 status and other clinicopathological parameters. Cases with incomplete p16 or microRNA data were excluded from the correlative analyses, resulting in a final matched cohort of 10 patients. One sample had already been excluded from the original microRNA analysis because it did not fulfil the quality-control criterion (mean miRTC - mean PPC ≤ 7). The present study has been independently approved by the Ethics Committee of Aristotle University of Thessaloniki (Protocol number 32272/14-Dec-2023). No new human participants were recruited, and no identifiable patient data are reported.

Tissue processing and microRNA profiling (previously published methods)

Paired samples of tumor and adjacent non-neoplastic tongue mucosa had been collected at the time of surgery, placed in RNAlater for 24 h at 4-8 °C, and stored at -80 °C until further processing. Total RNA, including small RNAs, was extracted with the miRNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Complementary DNA of mature miRNAs was synthesized using the miScript II RT Kit (Qiagen). Quantitative real-time PCR was performed with the miScript miRNA PCR Array (MIHS-102ZC, Qiagen) and the miScript

SYBR Green PCR Kit on an ABI 7500 Fast Real-Time PCR System under the following cycling conditions: 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 34 s, with subsequent melting-curve analysis. Data were analyzed with the SABiosciences miScript miRNA PCR Array Data Analysis software (SABiosciences Corporation, Frederick, MD, US) using the $2^{-\Delta\Delta Ct}$ method and the reference genes SNORD61, SNORD68, SNORD95, and SNORD96A for normalization.

p16 immunohistochemistry (new analysis)

Immunohistochemical staining for p16 was performed on 4-μm formalin-fixed paraffin-embedded sections of the same tumor specimens. A mouse monoclonal antibody against p16INK4A (clone IHC116; Abcam, Berlin, Germany) was used. Antigen retrieval was performed with heat-induced epitope retrieval according to the manufacturer's recommendations. The primary antibody was applied at a dilution of 1:200, followed by detection with a polymer-based horseradish-peroxidase system and diaminobenzidine chromogen on an automated staining platform. Positive controls (tonsil carcinoma) and negative controls (omission of primary antibody) were included in each staining run.

p16 immunoreactivity was evaluated by a pathologist blinded to the microRNA results. Cases were scored as p16-positive when moderate-to-intense nuclear and cytoplasmic staining was present in >70 % of neoplastic cells, and as p16-negative when staining was absent or limited to scattered/focal positivity within the neoplastic component. Staining of the adjacent non-neoplastic squamous epithelium was also recorded.

Statistical analysis

Associations between tumor microRNA expression and categorical clinicopathological variables (p16 status, sex, T-stage, tumor side, histological differentiation, hospital of origin and binary outcome: dead versus alive/disease-free) were examined with the Mann-Whitney U test (two groups) or the Kruskal-Wallis test (three groups). All group comparisons used the raw cycle-threshold (Ct) values obtained from the original miRNA PCR-array platform (lower Ct indicating higher expression); normalized ΔCt or $\Delta\Delta Ct$ values from the previously published tumor-versus-adjacent-normal analysis were not re-used for the p16 or clinicopathological comparisons. Correlation with age was assessed by Spearman's rank correlation coefficient. Approximate fold-change between groups was calculated as $2^{-(\Delta Ct)}$, where ΔCt = mean Ct of group 1 – mean Ct of group 2.

To preserve sensitivity in this small exploratory cohort, multiple-testing correction was performed separately within each clinicopathological variable (i.e., each variable constituted its own family of 84 tests) using the Benjamini-Hochberg false-discovery-rate (FDR) procedure. This approach treats each clinical question as independent and avoids an overly conservative global correction across all variables. A two-sided p-value < 0.05 was considered nominally significant; results remaining significant after FDR correction (FDR < 0.05) were regarded as statistically robust. Parallel analyses were also performed on the matched adjacent non-neoplastic tissue to assess tissue specificity. Given the limited sample size, all analyses were considered exploratory. Statistical computations were performed with Python (SciPy and statsmodels libraries).

Results

Patient characteristics of the matched cohort

Ten of the original 12 patients had complete data for both p16 immunohistochemistry and microRNA profiling and formed the final study cohort (Figure 1). There were six females and four males; age at diagnosis ranged from 37 to 76 years. Tumors were located on the left (n = 6) or right (n = 4) lateral border of the tongue. Histological differentiation was well differentiated in three cases and moderately differentiated in seven cases. T-stage was T1 in four patients and T2 in six patients. Six patients had died of disease, and four remained alive or disease-free at last follow-up. Table 1 summarizes the patients' characteristics.

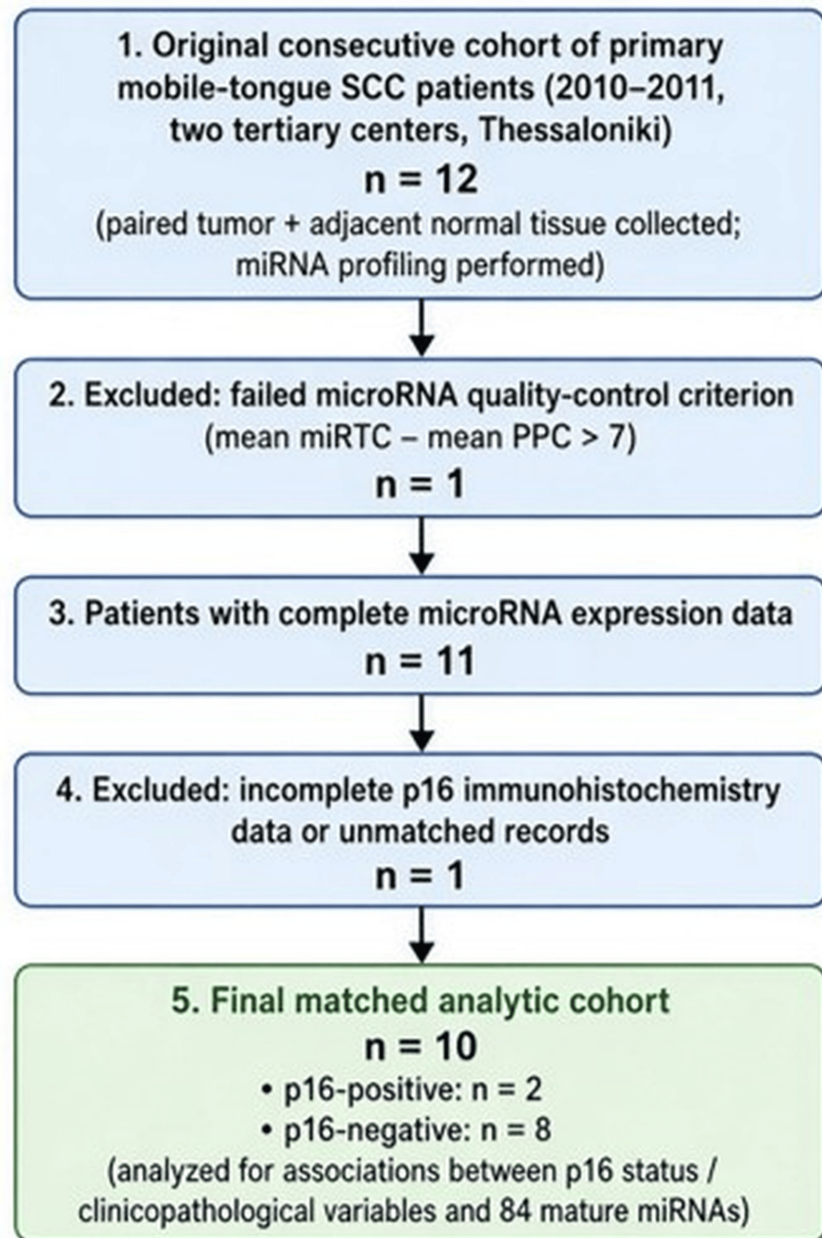


FIGURE 1: STROBE participant flow diagram

Patient	Gender	Age	Side	T-stage	Differentiation	p16 status	Outcome
1	Female	53	Left	T1	Well	Positive	DOD
2	Male	37	Left	T2	Moderate	Negative	DOD
3	Female	72	Right	T2	Moderate	Negative	DOD
4	Female	64	Left	T2	Well	Negative	DOD
5	Male	59	Right	T2	Moderate	Negative	NED
6	Male	47	Right	T1	Well	Negative	NED
7	Male	76	Left	T2	Moderate	Negative	DOD
8	Female	49	Left	T1	Moderate	Negative	NED
9	Female	53	Right	T1	Moderate	Positive	DOD
10	Female	70	Left	T2	Moderate	Negative	NED

TABLE 1: Clinicopathological characteristics of the matched cohort (n = 10)

DOD: died of disease; NED: no evidence of disease

p16 status

Two of the 10 tumors (20 %) were classified as p16-positive based on diffuse nuclear and cytoplasmic staining in ≥70% of neoplastic cells. The remaining eight tumors were p16-negative. Both p16-positive cases occurred in female patients who ultimately died of disease; one tumor was staged T1 (well differentiated) and the other T1 (moderately differentiated).

MicroRNA expression according to p16 status

Eighty-four mature human microRNAs were available for analysis in tumor tissue. Twelve microRNAs showed nominal differences between the two p16-positive and the eight p16-negative tumors (Mann-Whitney U test, uncorrected p = 0.044 for all twelve). After Benjamini-Hochberg FDR correction, none of these associations remained statistically significant (all FDR ≥ 0.31) (Table 2).

microRNA	Mean Ct p16+	Mean Ct p16–	ΔCt	Fold-change	Direction	p-value	FDR
hsa-miR-150-5p	20.63	23.90	–3.27	9.64	Higher in p16+	0.044	0.311
hsa-miR-206	23.58	26.37	–2.78	6.88	Higher in p16+	0.044	0.311
hsa-miR-155-5p	25.39	27.68	–2.29	4.89	Higher in p16+	0.044	0.311
hsa-miR-146a-5p	23.84	25.86	–2.01	4.04	Higher in p16+	0.044	0.311
hsa-miR-29b-3p	22.46	24.38	–1.92	3.79	Higher in p16+	0.044	0.311
hsa-miR-142-5p	26.25	27.37	–1.11	2.16	Higher in p16+	0.044	0.311
hsa-miR-140-5p	25.15	26.58	–1.43	2.70	Higher in p16+	0.044	0.311
hsa-let-7i-5p	22.06	23.22	–1.15	2.22	Higher in p16+	0.044	0.311
hsa-miR-205-5p	21.49	19.29	+2.20	0.22	Lower in p16+	0.044	0.311
hsa-miR-183-5p	30.02	27.72	+2.30	0.20	Lower in p16+	0.044	0.311
hsa-miR-135b-5p	27.49	24.99	+2.50	0.18	Lower in p16+	0.044	0.311
hsa-miR-200c-3p	23.89	21.96	+1.93	0.26	Lower in p16+	0.044	0.311

TABLE 2: MicroRNAs showing nominal differential expression according to p16 status in tumor tissue
Mann–Whitney U test (uncorrected)
FDR: Benjamini–Hochberg false-discovery rate; ΔCt: mean Ct of p16-positive – mean Ct of p16-negative
Fold-change calculated as 2^(-ΔCt). None of the associations remained significant after FDR correction.

The most pronounced nominal differences were higher expression in p16-positive tumors of miR-150-5p (approximate fold-change 9.6), miR-206 (6.9), miR-155-5p (4.9), miR-146a-5p (4.0), and miR-29b-3p (3.8), and lower expression of miR-205-5p, miR-183-5p, miR-135b-5p, and miR-200c-3p (fold-changes 0.18-0.26). miR-21-5p, which had been among the most strongly up-regulated microRNAs in the original tumor-versus-normal comparison (Paraskevopoulos et al., 2019), showed virtually identical expression between p16-positive and p16-negative tumors (fold-change ≈ 0.94, p = 0.89). Parallel analyses performed on the matched adjacent non-neoplastic tissue revealed no significant differences according to p16 status, indicating that the observed associations were tumor-specific.

Associations with other clinicopathological parameters

No microRNA demonstrated a significant association with patient outcome (dead versus alive/disease-free) after either uncorrected or FDR-corrected testing. A few nominal associations were detected, but none survived multiple-testing correction: sex - miR-138-5p (p = 0.019); sex - miR-138-5p and miR-378a-3p (p = 0.019 and 0.038); tumor side - miR-222-3p (p = 0.038). No significant associations were found with histological differentiation grade, hospital of origin, or age (Table 3).

Parameter	miRNAs tested	Nominal p < 0.05	FDR < 0.05	Comment
p16 status	84	12	0	Strongest (exploratory) signal
Sex	84	1	0	Only miR-138-5p (not robust)
T-stage (T1 vs T2)	84	2	0	miR-127-5p, miR-378a-3p (weak)
Tumor side	84	1	0	miR-222-3p (not robust)
Outcome (Dead vs Alive)	84	0	0	No association
Differentiation grade	82	0	0	No association
Hospital of origin	84	0	0	No association
Age (continuous)	82	0	0	No significant correlation

TABLE 3: Summary of associations between microRNA expression and clinicopathological parameters

FDR: Benjamini–Hochberg false-discovery rate

Discussion

The present analysis represents a direct continuation of our earlier miRNA profiling study of mobile-TSCC [21]. By re-examining the same archival specimens for p16 immunohistochemistry and integrating those results with the existing quantitative PCR array data, we sought to explore possible associations between p16 status--a widely used surrogate for transcriptionally active high-risk HPV infection--and the miRNA landscape of these tumors.

Only 2 of the 10 evaluable tumors (20%) were scored as p16-positive according to the stringent criterion of diffuse nuclear and cytoplasmic staining in ≥70% of neoplastic cells. This low prevalence is consistent with the literature indicating that true HPV-driven carcinogenesis is substantially less common in the mobile tongue than in the oropharynx [20]. Despite the small absolute number of p16-positive cases, 12 miRNAs showed nominal differences in expression between the p16-positive and p16-negative groups. The most striking numerical increases were observed for miR-150-5p, miR-206, miR-155-5p, miR-146a-5p, and miR-29b-3p, while miR-205-5p, miR-183-5p, miR-135b-5p, and miR-200c-3p appeared lower in p16-positive tumors. None of these differences survived formal multiple-testing correction, a finding that must be interpreted in light of the extremely limited statistical power inherent in comparing two versus eight samples.

Nevertheless, the nominal elevation of miR-155-5p in the p16-positive subset is noteworthy. miR-155 is one of the most extensively studied oncomiRs in OSCC; its overexpression has been linked to enhanced proliferation, metastasis, therapy resistance, and reduced survival in multiple independent cohorts [3,16,22]. Intriguingly, in HPV-positive oropharyngeal and tonsillar/base-of-tongue carcinomas, miR-155 expression has been reported to correlate with higher densities of CD8+ tumor-infiltrating lymphocytes and, in some series, with improved outcome [17,23]. Whether a similar immune-modulatory role operates in the rare p16-positive mobile-tongue tumors remains an open question that our data cannot resolve, but that warrants further investigation.

Other clinicopathological parameters, such as sex, T-stage, histological differentiation, tumor side, hospital of origin, and binary survival status, showed essentially no robust associations with the miRNA panel after correction for multiple testing. This null result is not surprising given the modest sample size and the biological heterogeneity of TSCC, but it does reinforce the relative specificity of the nominal p16-related signals.

A further point that merits emphasis is the paucity of published data that specifically address the relationship between p16 status and miRNA expression in mobile-TSCC, as opposed to oropharyngeal or mixed oral-cavity cohorts. Most available HPV-miRNA studies have focused on oropharyngeal tumors, where high-risk HPV is frequent, and p16 is a reasonably reliable surrogate [24,25]. In the mobile tongue, p16 overexpression is uncommon and often occurs in the absence of detectable HPV16, and dedicated correlative analyses of p16 (or HPV) with a broad miRNA panel remain scarce. Isolated reports have examined selected miRNAs together with p16 or HPV in oral dysplasia and mixed-site OSCC (e.g., miR-21, miR-22 and miR-224), but these cannot be extrapolated to a site-restricted TSCC cohort profiled with an 84-miRNA array [26,27]. The present pilot analysis therefore occupies a relatively unoccupied niche in the literature; its value lies less in confirmatory inference than in documenting this gap and nominating

candidates, notably miR-155-5p, for targeted validation.

Several important limitations must be acknowledged, in keeping with the STROBE and REMARK recommendations for observational and biomarker studies.

First, the effective sample size for the principal comparison (p16-positive versus p16-negative) was only two versus eight tumors. With such extreme imbalance, the Mann-Whitney test can detect only very large effect sizes, and the lowest attainable uncorrected p-value is constrained by the rank structure of the data (here 0.044 for all twelve miRNAs). Consequently, the identical nominal p-values largely reflect this rank structure rather than independent biological signals, and the twelve nominal associations are best regarded as hypothesis-generating rather than confirmatory. Fold-change estimates based on only two p16-positive tumors are inherently unstable; small shifts in the Ct values of either case can substantially alter the apparent magnitude of difference, so the numerical fold-changes reported in Table 2 should be interpreted with particular caution. Parallel analyses performed on the matched adjacent non-neoplastic tissue revealed no significant differences according to p16 status. While this is consistent with a tumor-restricted pattern, the absence of significance in adjacent tissue does not prove tumor specificity; the same severe sample-size constraints apply, and the analysis lacks power to exclude modest differences in non-neoplastic epithelium.

Second, p16 immunohistochemistry, while a practical and widely accepted surrogate for HPV status in the oropharynx, is imperfect in the oral cavity. False-positive p16 staining can occur through non-viral mechanisms (e.g., RB-pathway disruption), and conversely some transcriptionally active HPV infections may lack diffuse p16 overexpression. In the absence of direct HPV DNA or E6/E7 mRNA testing, we cannot definitively attribute the observed miRNA differences to viral oncogene activity.

Third, the original miRNA data were generated from fresh-frozen paired specimens using a commercial PCR-array platform and a fixed set of housekeeping genes. While this approach was appropriate for the 2019 discovery study, it does not allow absolute quantification or independent technical validation of individual candidates such as miR-155. Batch effects, RNA integrity differences, and the exclusion of one sample for quality-control reasons further constrain generalizability.

Fourth, the cohort is geographically and demographically homogeneous (two tertiary centers in northern Greece, 2010-2011). Lifestyle exposures (tobacco, alcohol, betel-nut) and the prevalence of high-risk HPV genotypes may differ substantially in other populations, limiting external validity.

Fifth, survival information was dichotomized (dead versus alive/disease-free) rather than analyzed as continuous time-to-event data with proper censoring. With only 10 patients and 6 events, formal Cox modeling was not feasible, and residual confounding by treatment, comorbidities, or stage cannot be excluded.

Finally, the study is purely correlative. No functional experiments (miRNA mimic/inhibitor transfection, target validation, or HPV E6/E7 modulation) were performed, so mechanistic claims cannot be advanced.

Despite these constraints, the work has several strengths: it re-utilizes a carefully annotated, previously published cohort; it applies transparent, non-parametric statistics with explicit multiple-testing correction; and it places the findings in the context of contemporary literature on miR-155 and p16 in oral cancer. The principal value of the present analysis therefore lies in generating prioritized candidates, especially miR-155-5p, for validation in larger, multi-institutional series that incorporate both p16 immunohistochemistry and direct HPV molecular testing, ideally accompanied by functional interrogation of the implicated miRNA-target networks.

Conclusions

In this exploratory extension of a previously characterized mobile-TSCC cohort, p16 status was associated with the largest number of nominal miRNA differences (12 miRNAs), while sex, T-stage and tumor side showed fewer nominal associations (1 or 2 miRNAs each). The most notable elevations in the two p16-positive tumors included miR-150-5p, miR-206, miR-155-5p, miR-146a-5p, and miR-29b-3p.

Twelve miRNAs, including miR-155-5p, showed identical nominal elevations or reductions in the two p16-positive tumors; none of these associations survived false-discovery-rate correction. The numerical findings are therefore hypothesis-generating only and do not support preferential biological inference for any single miRNA or for an HPV-related pattern (HPV status was not directly assessed). Larger multi-institutional series that combine rigorous p16 assessment with direct HPV testing and functional validation of nominated candidates are required before any of these signals can be interpreted as biologically or clinically meaningful.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Disclosures

Human subjects: Informed consent for treatment and open access publication was obtained or waived by all participants in this study. Ethics Committee of Aristotle University of Thessaloniki issued approval 32272/14-Dec-2023. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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