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Research Article

Circulating Cell-Free miR-154-5p in Plasma as A Non-Invasive Biomarker for Liquid Biopsy to Predict Survival in Oral Squamous Cell Carcinoma Patients

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Abstract

Earlier studies have demonstrated that clinical dysregulation of hsa-miR-154-5p expression was pathogenic in oral squamous cell carcinoma (OSCC). Currently, there is no blood-based molecular biomarker for risk stratification in OSCC. The present study aims to evaluate the level of circulating miR-154-5p expression in blood plasma for the first time as a potential biomarker for liquid biopsy that could be used for risk stratification and early prediction of the prognosis of OSCC patients. Blood samples (before surgery, CT, RT) were collected from each newly diagnosed OSCC patient with informed consent. In each patient, miR-154-5p expression levels were checked from cell-free blood plasma samples through qRT PCR. After surgery, the follow-up data was collected for the next two years. In the present investigation, during analysis, we first revisited the available datasets obtained from TCGA, dbDMEC database, CancerMIRNome database, and published literature sources and critically analyzed the clinical impact of miR-154-5p expression on OSCC. The results of qRT PCR revealed that miR-154-5p expressions in blood plasma samples were significantly downregulated ($p<0.0001$) (AUC of 0.900) in OSCC patients (n=25) compared to healthy control individuals (n=10). A comparative study was also performed to check the clinical association and correlation, between circulating miR-154-5p expression signature and conventional histopathological markers, and actual disease outcome (recurrence and survival) of each OSCC patient. Based on Mantel-Cox's survival analysis, the results revealed that OSCC patients with low miR-154-5p levels showed a shorter lifespan. Therefore, the downregulation of miR-154-5p is pathogenic in OSCC and this low level of circulating cell-free miR-154-5p could be a potential candidate biomarker for predicting poor survival in OSCC patients.

Keywords: Liquid biopsy, blood-based biomarker, circulating cell-free miRNA, prognosis, survival, OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent oral and maxillofacial malignancy, posing a significant threat in India. According to GLOBOCAN INDIA 2022, the most prevalent cancer among Indian men predominantly affects the gingiva-buccal region encompassing the buccal mucosa, retro-molar trigone, lower gum, tongue, and alveolus [1-3]. Smoking and chewing tobacco are the primary risk factors for OSCC, along with alcohol use, areca nut, betel leaf, and HPV infection. The disease is characterized by a high recurrence rate and alarmingly low (50%) survival rates. Surgery being the first line of treatment is followed by chemotherapy and radiotherapy [4-7]. Histopathological markers such as Lymphovascular Invasion (LVI), Perineural Invasion (PNI), Extranodal Extension (ENE), and Worst Pattern of Invasion (WPOI) are routinely evaluated and essential for risk stratifications to predict prognostic and therapeutic outcomes and to provide effective management strategies of OSCC [8, 6, 7, 9]. But they sometimes lack sensitivity and may produce false positives. The fatal outcome is primarily attributed to local recurrence, cervical (neck) lymph node metastasis, and distant organ metastasis [4, 10, 7, 11]. Distinguishing between a less-aggressive tumor with a favorable prognosis and a more-aggressive tumor with a poor prognosis, which exhibits therapy resistance and subsequently relapses and metastasizes, is a demanding task due to its diverse nature. Early detection of prognosis will facilitate treatment management. Notwithstanding advances in molecular diagnostics, no disease-specific blood-based molecular biomarkers are clinically available for early risk prediction of OSCC.

Previously, a couple of in-depth reviews have been conducted on the current knowledge and prospects of developing non-protein-coding RNA-based (miRNAs, lncRNAs, and so on) molecular diagnostics and therapeutics [12, 13]. Moreover, investigators critically analyzed the clinical significance of dysregulated non-coding RNAs like miRNAs and lncRNAs in tumor tissues and body fluids, to be a potential candidate for molecular biomarkers in OSCC diagnosis and prognosis [14, 12, 13]. Extensive research on miRNAs discovered that miR-154-5p expression is dysregulated in various types of malignancies such as NSCLC [15-17], breast cancer [18-20], skin squamous cell carcinoma [21], colorectal cancer [22, 23], prostate cancer [24], gastric cancer [25, 26], and cervical cancer [27, 28]. The miR-154 was first identified in idiopathic lung fibrosis in 2010 by Milosevic et.al, and demonstrated impactful tumor-suppressive effects in various human cancers and in 2020 first clinical trial was conducted on serum level of miR-154 (ChiCTR1800019872) to evaluate its diagnostic potential in patients with type 2 diabetes mellitus [15, 29]. Therefore, in this present investigation, we aimed to evaluate the clinical implications of circulating cell-free miR-154-5p expression in plasma as a potential blood-based molecular biomarker for risk stratification and early prediction of prognosis in OSCC patients. We also explored the clinical significance, association, and correlations between cell-free miR-154 levels in circulation and histopathological markers, and actual disease outcomes in OSCC in the Indian cohort.

Materials and Methods

Patient Recruitment and Sample Collection

In the current prospective patient cohort (sample size: 35), newly diagnosed OSCC patients were recruited from two hospitals (Tata Medical Centre or TMC and Netaji Subhas Chandra Bose Cancer Hospital or NSCBCH) in the same city, Kolkata in India. Wet lab experiments were performed at the respective hospitals' research wings (Tata Translational Cancer Research Centre or TTCRC and Netaji Subhas Chandra Bose Cancer Research Institute or NSCBCRI). 12 OSCC patients and 10 healthy controls individuals were recruited at NSCBCH, between 2021 and 2023 & 13 OSCC patients were recruited at TMC between 2017 and 2019. Patients with newly diagnosed primary OSCC and receiving treatment in the form of surgery with no known comorbidities. Blood samples were collected from healthy donors (n=10) and OSCC patients (n=25) (before surgery, CT, RT) with informed consent. Each patient received the standard care of treatment with curative intention in both hospitals. Each patient's treatment outcome data were collected during the follow-up period (two years after the surgery). The plasma were isolated from blood samples and used for this study. Circulating levels of miR-154-5p expressions were checked from cell-free blood plasma samples through qRT PCR. Ethics approval was obtained from the Institutional Ethical Committee (IEC) of both organizations following the approved guidelines.

In silico data collection and study plan

The expression data of miR-154-5p from clinical samples was collected from the dbDEMC (database of Differentially Expressed miRNAs in human Cancers) and CancerMIRNome. dbDEMC is an integrated database that designed to store and display differencially expressed miRNAs in 40 different types of cancers Bio-Med Big Data Center © 2016-2024 (BMDC). CancerMIRNome (jialab-ucr.org) is a comprehensive database with the human miRNome profiles of 33 cancer types from TCGA, NCBI GEO and ArrayExpress databases. The miR-154-5p expression data from five different datasets were collected – paired HNSCC and adjacent normal tissue samples (n = 15) $p<0.00148$ using Illumina miR arrays version (GEO Source ID: GSE31277), TCGA dataset of HNSC tissues (n = 523) and 44 normal tissues, $p< 1.69*10^-$

13 using limma miRNA seq data, NPC tissues (n=312) and 18 normal tissues, p<1.19*10^-4 using limma for microRNA profiling (GEO Source ID: GSE32960), paired OSCC and adjacent normal tissue samples (n = 40) p<2.99*10^-13, using Illumina Human v2 MicroRNA expression (GEO Source ID: GSE45238), Serum of OSCC patients (n = 25) and 15 healthy controls p<4.06*10^-4, using limma GEO (Source ID: GSE113956) in dbDEMC database. All these data are freely available online and do not require patient consent or authorization. The data is used without violating any individual or institutional rights. The circulatory levels of miR-154-5p were checked, correlated, and analyzed for their clinical implications in OSCC..

Sample collection, processing and storage

The peripheral blood (10ml) was collected in K2-EDTA tubes from each patient before surgery and healthy donor individuals after obtaining written informed consent. Plasma was separated by centrifugation at 2000 × g at 4°C within 6hrs hours after sample collection. The separated plasma was stored in aliquots at -80°C.1ml of Plasma from each sample was taken for RNA extraction from clinical samples. Detailed data on demographic characteristics, medical history, and clinical data including age, sex, tumour size, nodal status, TNM staging, histopathological findings, and follow-up information were meticulously documented from patient files. Detailed patient history and follow-up data are provided in the Table 1.

Sl. No.	Age	Gender	Primary sites at Diagnosis	Tumour Staging (AJCC 8th)	Tumour Grade (cell differentiation)	Whether in high-risk group	Lymphov ascular invasion	Perineural invasion	WPOI-5	Extranodal extension
1	40	M	OC T SCC	pT4aN2b	MDSCC	YES	present	present	present	absent
2	39	M	OC RBM SCC	pT3N0	WDSCC	NO	absent	absent	absent	absent
3	36	M	OC LBM SCC	pT4aN0	WDSCC	NO	absent	absent	absent	absent
4	33	M	OC T SCC	pT3N2a	MDSCC	YES	present	present	present	Present
5	37	M	OC LBM SCC	pT4aN3b	MDSCC	YES	present	present	absent	absent
6	43	M	OC LBM SCC	pT3N0	MDSCC	YES	absent	present	absent	absent
7	34	M	OC LBM SCC	pT4bN3b	MDSCC	YES	present	present	present	present
8	60	M	OC RBM SCC	pT3N0	MDSCC	NO	absent	absent	absent	absent
9	46	M	OC LBM SCC	pT4aN3b	MDSCC	YES	present	present	absent	present
10	49	M	OC LBM SCC	pT1N0	MDSCC	NO	absent	absent	absent	absent
11	47	M	OC RBM SCC	pT2N0	MDSCC	YES	present	present	present	absent
12	76	M	OC LBM SCC	pT4aN3b	MDSCC	YES	present	present	absent	present
13	56	M	OC ALV SCC	pT4aN0	MDSCC	YES	absent	absent	absent	absent
14	43	F	OC T SCC	pT2N2b	MDSCC	YES	absent	absent	absent	present
15	61	F	OC ALV SCC	pT4aN0	MDSCC	YES	absent	present	absent	absent

16	64	F	OC T SCC	pT3N2b	MDSCC	YES	present	present	present	absent
17	38	M	OC T SCC	pT3N1	MDSCC	NO	absent	absent	absent	absent
18	55	M	OC RBM SCC	pT3N1	MDSCC	YES	absent	absent	absent	absent
19	51	M	OC ALV SCC	pT3N0	MDSCC	YES	present	present	present	absent
20	59	F	OC T SCC	pT2N2b	PDSCC	YES	present	present	present	absent
21	60	F	OC RBM SCC	pT3N3b	MDSCC	YES	present	absent	absent	present
22	69	M	OC RBM SCC	pT3N0	WDSCC	NO	absent	absent	absent	absent
23	50	M	OC RBM SCC	pT3N3b	MDSCC	YES	present	present	absent	present
24	39	M	OC RBM SCC	pT4aN3b	MDSCC	YES	absent	absent	absent	absent
25	31	M	OC RBM SCC	pT4bN0	MDSCC	YES	absent	present	present	absent

Abbreviations
Gender: M-Male, F-Female,
Primary Sites: OC-Oral cancer, SCC- Squamous Cell Carcinoma, LBM- Left Buccal Mucosa, RBM-Right Buccal Mucosa, T-Tongue, ALV-Alveolus, Tumour Grade (cell differentiation): WDSCC-Well Differentiated Squamous Cell Carcinoma, MDSCC-Moderately Differentiated Squamous Cell Carcinoma, PDSCC-Poorly Differentiated Squamous Cell Carcinoma.

Table 1: Represents the clinicopathological details of the 25 OSCC patients.

RNA Isolation & cDNA Preparation

Total RNA was isolated from 1ml plasma using the TRIzol LS reagent (Invitrogen). 50 ng RNA was used for cDNA synthesis using miScript II RT kit (Qiagen). Then, 100 picogram cDNA was used for the miRNA quantification.

miRNA Quantification by Real-Time PCR (qRT-PCR) and RT-PCR

The miR-154-5p expression was checked through quantitative real-time PCR using miScript SYBR Green PCR kit (Qiagen) following the manufacturer's protocol with miR-154-5p specific miScript primers assays. Here, miR-16-5p was used as an endogenous control with miR-154-5p specific miScript primers assays. All reactions were conducted in duplicate with two biological repeats. The CT value (cycle threshold) was considered only if the value was <38 for miR-154-5p and <30 for miR-16-5p, indicating the number of cycles needed for the fluorescent signal to cross the threshold in qPCR. ΔCT and ΔΔCT, the fold change (FC), and finally, the relative fold change (RFC) were calculated as the expression levels of miRNA.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism version 8 (GraphPad Software, Inc., La Jolla, CA, USA). The expression level of miR-154-5p of healthy controls and among the different subgroups of OSCC patients were compared using non-parametric tests such as Mann-Whitney's test and Krushkal-Walli's comparisons test, respectively. A p-value of less than 0.05 was considered statistically significant. Mann–Whitney U test was used to compare different miRNA levels among various groups of OSCC patients, with data presented as a median with 95% confidence interval (CI). The ROC curve of miR-154-5p relative fold change in OSCC patients against healthy controls was constructed to determine the diagnostic potential of miR-154-5p for OSCC detection. Log-rank (Mantel-cox's) test and the Gehan-Breslow-Wilcoxon survival analysis were used to analyze the disease-free survival and overall survival of OSCC patients having high or low miR-154-5p expression based on the average cut-off values.

Results

Identification of miR-154-5p as a potential candidate for validation as a prognostic biomarker in OSCC

Initially, we conducted a comprehensive literature review and systematic analysis to identify miR-154-5p as a potential biomarker for OSCC. Following this, we examined its role in OSCC by analyzing tissue and blood samples from OSCC patients using data sourced from relevant databases. To further elucidate the involvement of miR-154-5p in the initiation, progression, and recurrence of OSCC, we systematically aggregated and analyzed data from multiple literature sources that describe its association with OSCC. Our analysis revealed consistent downregulation of miR-154-5p expression in tissues from oral squamous cell carcinoma (OSCC), head and neck squamous cell carcinoma (HNSCC), nasopharyngeal carcinoma (NPC), glioma, adenoid cystic carcinoma (ACC), and oropharyngeal squamous cell carcinoma (OPSCC) relative to adjacent normal tissues. These findings suggest a potential tumor suppressor role of miR-154-5p in the development of these carcinomas (Table 2). Next, the consequences of data analysis of miR-154-5p expression obtained from the dbDEMC database demonstrated the downregulation of miR-154-5p in HNSCC [TCGA, GSE31277], NPC [GSE32960], and OSCC [GSE45238] tissues compared to adjacent normal tissues, suggesting its role as a tumor suppressor and potential diagnostic biomarker in OSCC (Figure 1.A). However, an additional dataset [GSE113956] revealed upregulation of miR-154-5p in serum samples from OSCC patients compared to healthy controls (Figure 1.B). In the current study, we have explored the clinical role of miR-154 expression levels in blood plasma for its diagnostic and prognostic significance in OSCC.

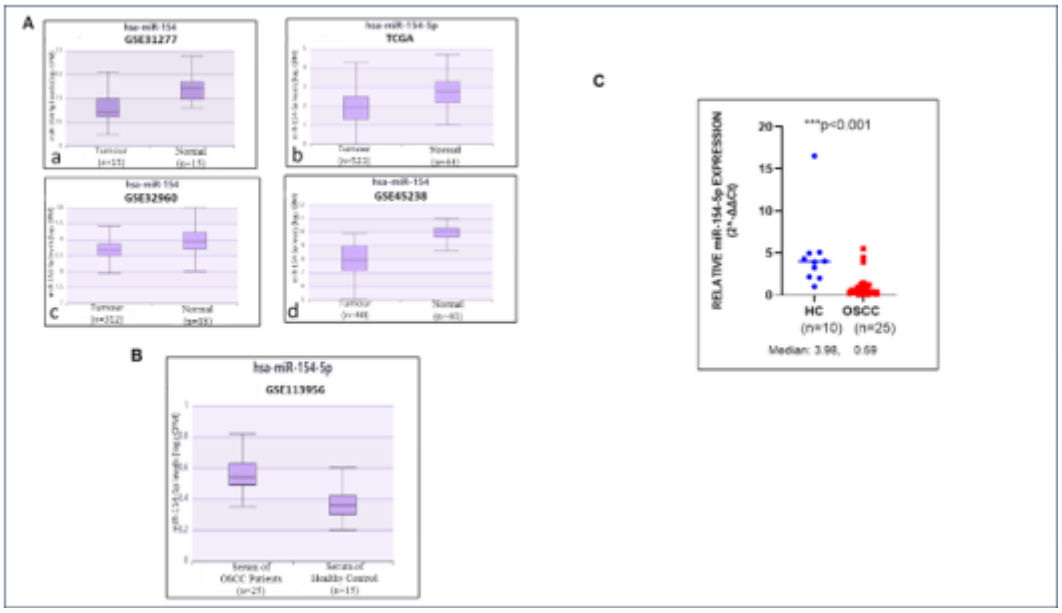

Figure 1: A. The expression data of miR-154-5p in tumour tissues and normal tissues of patients obtained from the dbDEMC and CancerMIRNome databases: (**a)** In a study of paired HNSCC and adjacent normal tissue samples (n = 15) p<0.00148 using Illumina miR arrays version, GSE31277, (**b**) In the TCGA dataset of HNSC tissues (n = 523) and 44 normal tissues, p< 1.69*10^-13, using limma miRNA seq data, (**c**) In the group of NPC tissues (n=312) and 18 normal tissues, p<1.19*10^-4, using limma for microRNA profiling, GSE32960, (**d**) In a study of paired OSCC and adjacent normal tissue samples (n = 40) p<2.99*10^-13, using Illumina Human v2 MicroRNA expression, GSE45238; **B.** A study from serum of OSCC patients (n = 25) and 15 healthy controls p<4.06*10^-4, using limma, GSE113956. **C.** In our prospective study: Comparison of normalized relative expression of miR-154-5p in oral squamous cell carcinoma (OSCC) patients (n=25) and the control group (n=10).

CANCER TYPE	CLINICAL SAMPLES	CELL LINES	TARGET MOLECULE/ PATHWAY	EFFECT OF miR-154	REFERENCES
Oral squamous cell carcinoma (OSCC)	Matched Oral cancer tissues and adjacent normal tissues (n=5)	Ca9-22, HSC-2, HSC-3, and HSC-4 cells	HDAC1 regulated expression of PCNA	HDAC1 can negatively regulate the expression of miR-154-5p, an inhibitor of miR-154-5p can attenuate PCI24781 treatment decreased PCNA expression and cell proliferation	[30]
Head and neck squamous cell carcinoma (HNSCC)	The TCGA expression data of miR-154-5p in HNSCC tissues (n=497) and normal tissues (n=44), GEO datasets of HNSCC tissues (n=30 and n=71)		Upregulation of miR-154-5p was associated with the upregulation of Oncogenic Pathways in the HNSCC Patients	miR-154-5p up-regulation correlated with better immune response. It also can be used as a biomarker describing clinical, molecular, and immunological features of HNSCC patients.	[31]
Nasopharyngeal carcinoma (NPC)	Frozen NPC tissue(n=40) and normal nasopharyngeal epithelial tissues (n=8)	NPC cell lines (5-8F, SUNE-1, 6-10B, CNE-1, CNE-2, HNE-1 and C666-1) and human immortalized nasopharyngeal epithelial cell lines (NP69)	Kinesin-like protein KIF14 was identified as a new functional target of miR-154-5p	MiR-154-5p binds to the 3′-UTR of KIF14 mRNA and decreases the expression of KIF14 protein, suppressing NPC migration and metastasis.	[32]
Oral squamous cell carcinoma (OSCC)	The GEO dataset GSE45238 containing OSCC tissue (n=80) and their adjacent non-tumour epithelium, based on the GPL8179 platform		miRNA target genes CALM1, CYCS, THBS1, MYC, GATA6, PIK3R3, GIGYF1, BCL2L11, SPRED3 were assessed to predict prognosis	Out of the 23 miRNAs, miR-154-5p was identified to be under expressed in early OSCC tissues compared to healthy controls	[33, 34]

Adenoid cystic carcinoma (ACC)	19 salivary adenoid cystic carcinoma patients	none	miRNA expression analysis in salivary gland ACC tumour samples using nCounter Technology (NanoString Technologies, Seattle, WA, USA)	Widely associated with tumour suppression, migration, invasion, and metastasis processes in several tumour types, supporting the relationship with the more aggressive behaviour of this growth pattern type.	[35]
Oropharyngeal squamous cell carcinoma (OPSCC)	e22 HPV (+) patients (n=9) current or past smokers and (n=13) never smokers	OPSCC tissues	HNSCC cell line HPV (-) UMSCC11A HPV (+) cells UMSCC47 and HPV (-) SCC1A	miRNA expression landscape in HPV (+) smokers and non-smokers in OPSCC tissues HuR and EGFR pathways	[36]

Table 2: miR-154-5p dysregulation in clinical samples, cell lines, target molecules and their clinical consequences in Oral Cancer.

Validation of Circulating miR-154-5p levels in the plasma of OSCC patients and healthy controls

To evaluate the clinical significance of miR-154-5p, we conducted a prospective study comparing plasma samples from treatment-naïve OSCC patients with plasma samples from healthy controls. Circulating level of miR-154-5p expression was quantified using quantitative real-time PCR (qRT-PCR), with results normalized to an endogenous control (miR-16-5p). Statistical analysis via the Mann-Whitney U test demonstrated a significant reduction in circulating miR-154-5p levels in plasma samples of OSCC patients compared to healthy controls ($p<0.0001$) (Figure 1.C). This observed downregulation in plasma parallels findings from tissue datasets and supports the hypothesis that miR-154-5p functions as a tumor suppressor in OSCC. These results emphasize the potential of miR-154-5p as a robust biomarker for OSCC detection, with our findings aligning closely with data from tumor tissue-based existing databases, thereby suggesting that miR-154-5p expression in plasma reciprocated the tumor-associated miR-154-5p in OSCC samples.

Evaluation of circulating miR-154-5p levels based on the Clinicopathological Parameters

The study compared the relative miR-154-5p expression levels in OSCC patients with different lymph node metastasis statuses. First, the miR-154-5p levels in plasma were compared and evaluated for the presence or absence of metastatic lymph nodes N(+) and N0 in OSCC patients to assess the risk of recurrence using the Mann-Whitney test. The study did find a noticeable trend indicating the presence of lower levels of miR-154-5p in patients with positive lymph nodes compared to the node-negative patients (Figure 2.A). In the conventional method, OSCC patients were stratified into high-risk and low-risk groups based on the presence or absence of some histopathological markers: LVI, PNI, ENE, and WPOI-5. High-risk patients had anyone or more markers positive, while low-risk patients had none. The current study investigated the association between the level of miR-154-5p and specific histopathological markers. The analysis showed a consistent trend of reduced miR-154-5p levels in patients with these markers. The median relative fold change of miR-154-5p was found to be lower in high-risk patients compared to low-risk patients (Figure 2.B). This suggests that reduced miR-154-5p levels may be associated with more aggressive disease characteristics in OSCC patients. Since PNI, LVI, ENE, and WPOI-5 are known markers of poor prognosis, the lower expression of miR-154-5p in patients with these features implies that it could play a role in tumor progression and invasion. Low level of miR-154-5p in plasma might serve as a potential biomarker for identifying high-risk OSCC cases, aiding in prognosis and treatment decisions.

Furthermore, miR-154-5p might serve as a potential biomarker for identifying high-risk OSCC cases, aiding in prognosis and treatment decisions. This finding also raises the possibility that miR-154-5p could be involved in the biological mechanisms underlying OSCC aggressiveness, making it a potential target for future therapeutic interventions.

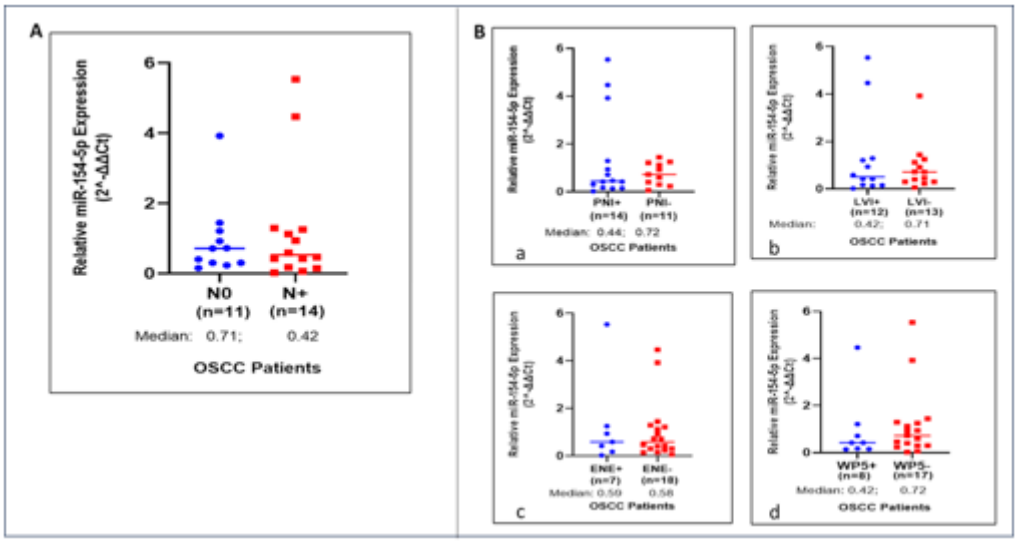

Figure 2: A. Shows the relative expression of miR-154-5p in OSCC patients (n = 25) with different lymph node metastasis status indicating the presence N(+) and absence N0 of metastasis using MannWhitney test p<0.69. B. Expression of miR-154-5p in different groups of patients (n = 25) analyzed using Mann Whitney test. OSCC patients were categorized according to: (**a**) Presence and absence of Perineural Invasion: PNI+ vs PNI- patients (p<0.81), (**b**) Presence and absence of Lymphovascular Invasion: LVI+ vs LVI- patients (p<0.88), (**c**) Presence and absence of Extranodal Extension: ENE+ vs ENE- patients (p<0.98), (**d**) Presence and absence of Worst Pattern of Invasion-V: WPV+ vs WPV- patients (p<0.43).

Trends in miR-154-5p levels based on the Clinical TNM staging

We conducted a comparison of miR-154-5p expression between clinical Stage I and II OSCC and Stage III and IV OSCC. We aimed to determine whether miR-154-5p is involved in the advancement or progression of OSCC. The findings revealed remarkably low miR-154-5p expression in Stage III & IV patients compared to Stage I & II patients, ascertaining its role in the advancement or progression of OSCC (Figure 3.a). The observed correlation between miR-154-5p levels and OSCC stage might prove its importance as a marker for monitoring disease progression.

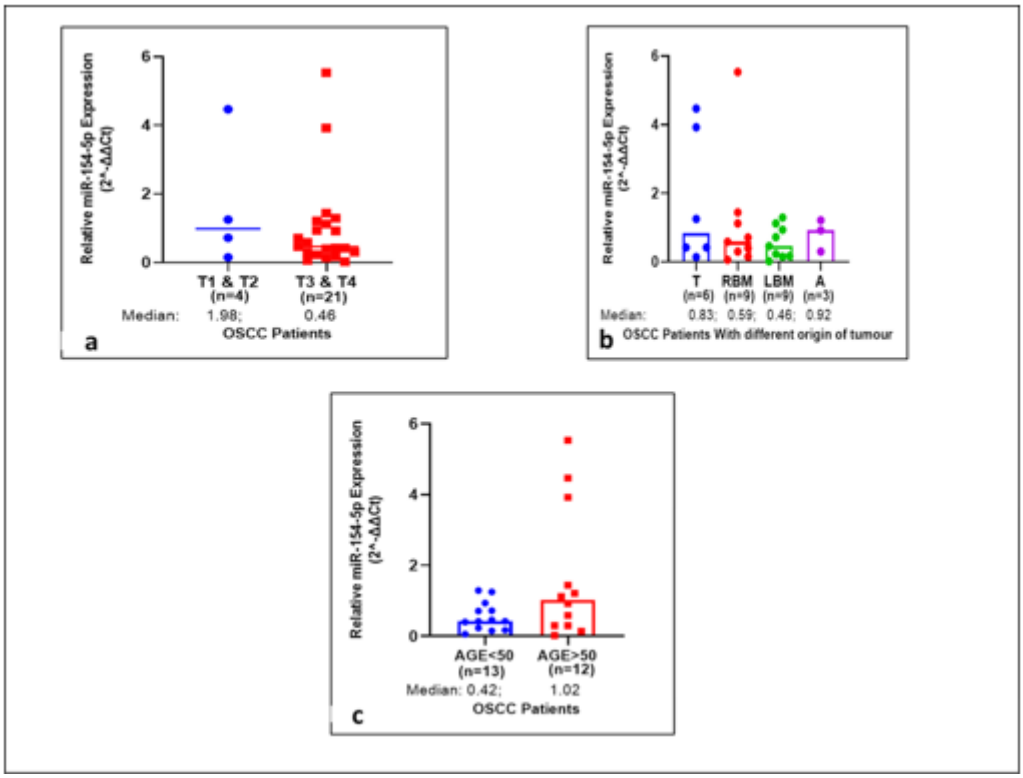

Figure 3 (**a**): Expression of miR-154-5p in OSCC patients (n = 25) with different clinical stages. The expression of miR-154-5p was compared between T1 &T2 stage patients vs T3 & T4 patients and compared using Mann Whitney test p<0.44, (**b**) Expression of miR-154-5p in OSCC patients (n = 25) among patients with different subsites of origin of OSCC. The expression of miR-154-5p was compared between subsites such as tongue, right buccal mucosa (RBM), left buccal mucosa (LBM), and Alveolus in OSCC patients using Krushkal Wallis test, p<0.44. (**c**) Expression of miR-154-5p in OSCC patients (n = 25) with different age groups. OSCC patients aged <50 years were compared to patients >50 years using the Mann-Whitney test p<0.44.

Different subsite-based miR-154-5p expression

Next, we categorized patients based on the origin of the tumor from different subsites and compared the levels of miR-154-5p using the Kruskal-Wallis comparative test to identify the most common site of origin of OSCC. The results emerged with lower median miR-154-5p levels in the left buccal mucosa, right buccal mucosa, tongue, and alveolus, respectively. This finding indicates buccal mucosa might be the most common site of origin of OSCC, based on our study (Figure 3.b). Subsite-specific differences in circulatory miR-154-5p levels may warrant its contribution to site-specific factors. Further research is needed to explore the underlying mechanisms driving this site preference and to validate miR-154-5p as a potential biomarker for OSCC detection and prognosis across different anatomical locations.

Age-based analysis of miR-154-5p levels among OSCC patients

To understand the potential age of OSCC onset, we compared and analyzed the levels of miR-154-5p among OSCC patients grouped into those under 50 years and those over 50 years. The findings suggested a noticeable decrease in miR-154-5p expression levels among patients under 50 years old compared to those over 50 years old suggesting the ideal age of OSCC onset to be less than 50 years (Figure 3.c). The age-related decline in miR-154-5p expression may suggest its potential role in the early onset of OSCC and its implications for age-specific screening and prevention strategies.

Prediction of prognosis

In the present investigation, our primary aim was to pinpoint early indicators that could forecast the prognosis of patients with OSCC. To accomplish this, we categorized the patients into two distinct groups based on actual outcome data (two years of follow-up): those who experienced a recurrence and those who did not. We then employed the Mann-Whitney test to conduct a thorough comparative analysis of miR-154-5p levels between these two groups. Notably, some of the recurrent patients in the study also presented with metastasis. The consistent decrease in miR-154-5p levels in recurrent patients compared to non-recurrent ones indicates its potential role as a prognostic biomarker for disease recurrence (Figure 4.A). Lower miR-154-5p expression could be linked to more aggressive tumor behavior, increased invasiveness, or resistance to treatment, making it a valuable target for further investigation. Understanding this association could aid in identifying high-risk patients early, allowing for closer monitoring and tailored therapeutic strategies to improve outcomes.

Evaluation of the Diagnostic Potential of miRNA miR-154-5p Signature for OSCC

The ROC curve of miR-154-5p was generated to unfold the diagnostic performance of miR-154-5p in OSCC. The relative fold change in OSCC patients against healthy controls generated an AUC of 0.900, 95% CI - 0.7958 to 1.000, and a p-value of 0.0003 (Figure 4.B). Hence, it determines that miR-154-5p has significant diagnostic potential. The strong diagnostic performance of miR-154-5p suggests its potential as a non-invasive discriminatory biomarker of OSCC. However, to confirm its clinical utility, further studies are needed to validate these findings across diverse populations and clinical settings.

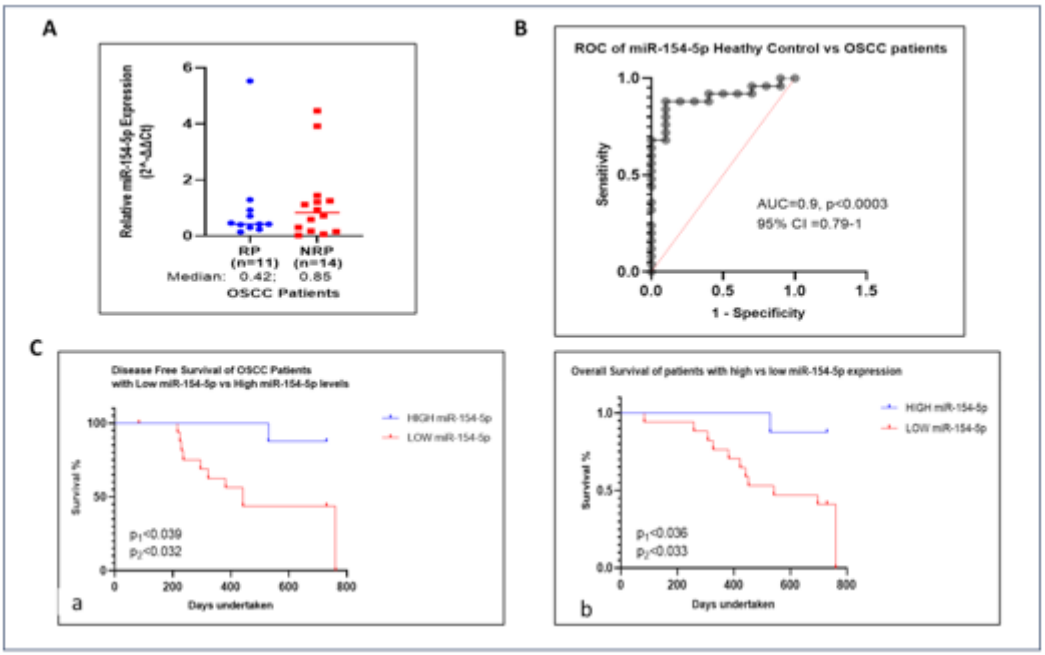

Figure 4: A) Shows the relative expression of miR-154-5p in Recurrent vs Non-recurrent OSCC patients (n = 25) using Mann-Whitney test p<0.65. **B**) Receiver operating characteristic (ROC) analysis for miR-154-5p indicating a relatively higher area under the curve (AUC=0.90), p<0.0003 calculated using Wilson/Brown test, miR-154-5p was found to have high diagnostic potential, **C**) Survival analysis of OSCC patients (n=25) having relatively high miR-154-5p vs low miR-154-5p expression using Log-rank (Mantel-Cox) survival analysis (p1), Gehan-Breslow-Wilcoxon test (p2): (**a)** Disease-Free Survival of OSCC Patients: A significant increase in recurrence is seen in patients with low miR-154-5p expression levels compared to patients with high miR-154-5p expression levels, p1<0.039, p2<0.032, (**b**) Overall survival of OSCC Patients: A noticeable increase in mortality rate was observed in patients with low miR-154-5p expression levels compared to patients with high miR-154-5p expression levels, p1<0.036, p2<0.033.

Survival analysis of the OSCC patients based on high or low miR-154-5p levels

The patients were separated into two groups according to their miR-154-5p expression levels, with the average expression serving as a threshold (Table 3). The 2-year Disease Free Survival (DFS) and Overall Survival (OS) were then evaluated using Kaplan Meier analysis and compared statistically using the Log-rank (Mantel-Cox) survival analysis (p1) and the Gehan-Breslow-Wilcoxon test (p2). Patients with high miR-154-5p expression levels had substantially longer DFS than those with low levels ($p1<0.039$, $p2<0.032$) (Figure. 4.C). Patients with high miR-154-5p expression levels had a significantly higher OS than those with low levels ($p1<0.036$, $p2<0.033$) (Figure 4.C). Therefore, miR-154-5p has the potential to be integrated into prognostic models for identifying patients at heightened risk of poor outcomes, which could lead to more tailored and aggressive therapeutic interventions.

	miR-154 expression		
	LOW EXPRESSION N (%)	**HIGH EXPRESSION N (%)**	**TOTAL (N=25)**
Average Age	25		
Gender Ratio			
MALE	16	7	23 (92%)
FEMALE	1	1	2 (8%)
Primary Site			
LBM	7	2	9 (36%)
RBM	6	3	9 (36%)
T	3	3	6 (24%)
ALV	2	1	3 (12%)
Nodal Status			
N0	8	3	11 (44%)
N+	9	5	14 (56%)
Tumour Staging			
T1	1		1 (4%)
T2	1	2	3 (12%)
T3	6	5	11 (44%)
T4	9	1	10 (4%)
Risk Grade			
High-risk	11	7	18 (72%)
Low-Risk	6	1	7 (28%)
Histo-path Markers			
LVI+	8	4	12 (48%)
LVI-	9	4	13 (52%)
PNI+	10	4	14 (56%)
PNI-	7	4	11 (44%)

ENE+	5	2	7 (28%)
ENE-	12	6	18 (72%)
WP5+	6	2	8 (32%)
WP5-	11	6	17 (68%)
Outcome Data: June'24			
RECURRENT	10	1	11 (44%)
NON-RECURRENT	7	7	14 (56%)
METASTATIC	10	1	12 (48%)
NON-METASTATIC	7	7	13 (52%)
Status			
DEAD	10	1	11(44%)
ALIVE	7	7	14 (56%)

Table 3: Represents the age and classification statistics of 25 OSCC Patients with high and low miR-154-5p expression levels.

Discussion

OSCC presents a significant clinical challenge, with a five-year recurrence rate of around 50%, particularly within the first two years following treatment [37]. Tumor tissue biopsy and histopathological analysis are the gold standard for assessing risk in OSCC patients [38]. Despite advancements in molecular diagnostics, there remains a dearth of specific biomarkers capable of predicting OSCC risk at an early stage. The circulating cell-free miRNAs are particularly fascinating for clinical applications because of their remarkable stability in the bloodstream, attributed to protective modifications such as uridylation, adenylation, and methylation and their encapsulation in exosomes, micro-vesicles, and apoptotic bodies, along with their binding to RNA-binding proteins, making them ideal candidates for diagnostic tools [39-42]. Recently, in a clinical trial (ChiCTR1800019872), miR-154 expression signature in blood serum was investigated for its diagnostic potential overcoming numerous challenges in type 2 diabetes mellitus patients [29]. However, the potential of cell-free miR-154-5p as a biomarker for OSCC detection and prognosis in OSCC remains unexplored, while most research focuses on malignant cell lines and clinical tissue specimens. The current study elucidates the role of miR-154-5p as a significant biomarker directly from plasma samples for liquid biopsy in OSCC, highlighting its potential for diagnosis, prognosis, and disease progression. The present study demonstrated a significant reduction in circulating miR-154-5p levels in plasma samples of newly diagnosed treatment-naïve OSCC patients compared to healthy controls (Fig. 1C). The results strengthen its diagnostic potential and pattern support its tumor suppressor role in OSCC. This finding is corroborated with previous studies discovered by analyzing the datasets of HNSCC [TCGA, GSE31277], NPC [GSE32960], and OSCC [GSE45238] from the dbDEMC database using tumor tissues indicating lower miR-154-5p levels in OSCC. This suggests tumor tissue-specific molecular analytes carry forward to the circulation that reciprocates responses in the plasma of blood circulation. The upregulation of miR-154-5p in the serum of OSCC patients was revealed by an independent dataset [GSE113956], which was probably acquainted with a compensatory mechanism in the circulation with the tumor-associated expression profile of OSCC patients [43]. The clinical relevance of miR-154-5p in OSCC diagnosis and prognosis should be investigated through comprehensive studies that integrate tissue and circulating miRNA profiles. In the current scenario, several studies revealed that cell-free miRNAs in circulation hold significant potential as minimally invasive biomarkers for early detection of prognosis, disease monitoring, and for treatment (immunotherapy and/or chemotherapy) response, emphasizing the importance of advanced technologies to optimize their clinical utility as declared by other several investigators [44-46, 40].

The present investigation explored the relationship between miR-154-5p expression and various clinicopathological parameters, including nodal status, histopathological markers (LVI, PNI, ENE, WPOI-5), clinical TNM staging, tumor subsite, and patient age. The consistent downregulation of miR-154-5p in patients with high-risk histopathological markers and advanced TNM stages suggests its involvement in OSCC progression. Similar observations were described in patients with bladder cancer, where miR-154 downregulation was significantly associated

with advanced tumor staging, higher histologic grades, and lymph node metastasis [47]. Additionally, the lower expression of miR-154-5p in younger patients (<50 years) compared to older patients suggests that miR-154-5p may be associated with early-onset OSCC. Prospective studies should further investigate miR-154-5p's role in these contexts, which could lead to more personalized treatment approaches. In terms of prognosis, our analysis revealed that lower miR-154-5p levels are associated with OSCC recurrence and metastasis, indicating its potential as a prognostic marker. This finding is crucial as it suggests that miR-154-5p could be used to predict the likelihood of recurrence and metastasis in OSCC patients, potentially guiding more tailored therapeutic interventions. The diagnostic potential of miR-154-5p was further evaluated through ROC curve analysis, which yielded an AUC of 0.900, indicating high diagnostic accuracy. This result suggests that miR-154-5p could serve as a non-invasive biomarker for OSCC detection, providing a valuable tool for early diagnosis. However, further validation in diverse populations is required to confirm its applicability in clinical settings. Finally, survival analysis based on miR-154-5p expression levels revealed that patients with higher miR-154-5p levels had significantly longer Disease-Free Survival (DFS) and Overall Survival (OS). Research in human glioma, melanoma, and bladder cancer patient survival has supported these findings [48, 49, 47]. This finding suggests that miR-154-5p could be incorporated into prognostic models to identify patients at higher risk of poor outcomes, enabling more personalized and intensive therapeutic approaches. Future research should focus on validating these findings and exploring the feasibility of integrating miR-154-5p into clinical practice, particularly in the context of personalized medicine.

Conclusion

In conclusion, this multicentered study demonstrated the clinical significance of circulating miR-154-5p levels in blood plasma in OSCC, demonstrating its potential as a biomarker for diagnosis, risk stratification, and prediction of survival of the patients with OSCC. The results suggested that circulating level of miR-154-5p could predict the survival (DFS and OS) significantly in OSCC and therefore, it could be a potential liquid biopsy biomarker to predict survival in OSCC patients. Understanding whether blood levels of miR-154-5p accurately reflect disease activity or response to treatment will be essential for integrating this miRNA into clinical practice. Therefore, further study with a larger patient cohort is essential to validate these findings and explore the mechanisms underlying miR-154-5p's role in OSCC. This could lead to significant advancements in managing OSCC malignancy.

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Institutional Review Board Statement: The study was approved by the Institutional Ethical Committee (IEC) (Approval letter dated 15.12.2021) of NSCBCRI and the Institutional Review Board (IRB) (Ref No. EC/GOVT/12/16 dated 06.01.2017) of the TTCRC and carried out following the approved guidelines. Patients and healthy control individuals were recruited in the study after obtaining informed written consent and willingness to donate their blood samples for this study.

Data Availability Statement: All the data are available online with common access. The data analyzed during this study are available from the corresponding author upon reasonable request. Data supporting reported results can be found in dbDEMC database and CancerMIRNome databases.

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Abbreviations

The following abbreviations are used in this manuscript:

OSCC-Oral Squamous Cell Carcinoma

miRNA-microRNA

lncRNA- long non-coding RNA

TCGA- The Cancer Genome Atlas Program

dbDEMC- database of Differentially Expressed miRNAs in human Cancers

qRT PCR- quantitative Real-Time PCR

LVI- Lympho-vascular Invasion

PNI- Perineural Invasion

ENE- Extra-Nodal Extension

WPOI- Worst Pattern of Invasion

HNSCC- Head and Neck Squamous Cell Carcinoma

NPC- Naso-Pharyngeal Carcinoma

ACC- Adenoid Cystic Carcinoma

OPSCC- Oro-Pharyngeal Squamous Cell Carcinoma

DFS- Disease-Free Survival

OS- Overall Survival

LBM- Left Buccal Mucosa

RBM- Right Buccal Mucosa

T- Tongue

A- Alveolus

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