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The Role of Salivary MicroRNAs as a Diagnostic Marker in Early Detection of Oral Squamous Cell Carcinoma

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Author`s Contribution

[1]Conception and design, drafting of the manuscript
[2]Guidance and revision of the manuscript and critical analysis.
[3]Data analysis and collection.
[4]Material preparation and literature search.[5,6]Drafting the work or revising it critically for important intellectual content

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ABSTRACT

Objective To assess the diagnostic role of salivary microRNA for early detection of oral squamous cell carcinoma and to compare the salivary microRNA with Biopsy in the diagnosis of oral squamous cell carcinoma.

Methodology: This descriptive cross-sectional study was conducted in the department of pathology and molecular laboratory, department of oral & maxillofacial surgery of Liaquat University of Medical & Health Sciences, Jamshoro / Hyderabad from March 2022 to August 2022. All histopathologicaly diagnosed OSCC patients of both genders with age group 18 years and above will be included in the study after taking their informed consent. Whole saliva samples were collected after avoid eating, drinking, smoking or oral procedures for at least 1 h prior to the collection of samples. After five minutes of oral rinsing spit 5 mL of saliva into a 50 mL sterile tube placed on the ice. The tube should remain on ice while collecting the saliva samples. Four hundred microliters of the whole saliva mixture (200 µL whole saliva and 200 µL RNA later), and 400 µL of the supernatant saliva was used for RNA extraction. The data was entered and analyzed.

Results: Mean age of the patients was 47.45+10.85 years. 85(70.8%) were married and 35 (29.2%) were unmarried. 90.0% were married and 5.0% were unmarried, while 5.0% were widow. Cigarette smoking, paan, chaalia and naswar were the commonest cause of the oral squamous cell carcinoma. Most of the cases 59.2% had moderately differentiated SCC, 30.0% cases had well-differentiated SCC and 10.8% of the cases had poor differentiated SCC. Out of all study subjects, 87.5% of the patients had positive micro-RNA expression. Micro-RNA expression was significantly associated, with poorly differentiated squamous cell carcinoma (p=0.016).

Conclusion: Study revealed that the salivary miroRNA expression were significantly positive in patients of OSCC. Therefore, salivary microRNA could be considered as a useful and advantageous biomarker for the detection and tracking of OSCC across various levels of tissue abnormalities.

Key words: *OSCC*, Micro-RNA, Diagnosis, Expression

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Introduction

Oral Squamous Cell Carcinoma (OSCC) is a priority public health problem worldwide.[1] It ranks sixth in the list of most prevalent cancers of oral cavity globally.[1] As estimated by World Health Organization, OSCC cause the highest mortality rate as compared to other cancers with a death rate of about 45% from five years after the

diagnosis.[2] Greater than 90% of all cases of this fatal disease can involve any area of oral cavity including the labial and buccal mucosa, the anterior two-thirds of the tongue, the retro molar pad, the floor of the mouth, the gingiva and the palate. The burden of new cases is rising particularly in the poor and middle socioeconomic countries. Its higher frequency in south and South East Asian countries including Pakistan has been well recorded.[3] The cancer of the oral cavity and pharynx are amongst the commonest type of cancers. As stated by PMRC, it is the most common cancer among males and second highest to breast in females.[4] Despite significant advancements in oral cancer prevention, the survival rate for OSCC has remained stagnant at up to 50% for a 5-year period.[5]The five year survival rate can be enhanced near to 80%if it is diagnosed at the earlier stages (T-1), Whereas the disease in advanced stage (stage III-IV), the prognosis drop to 30-50%.[6] .Delay in diagnostic, metastasis to regional lymph nodes and reoccurrence are the main factors that lead to poor prognosis, higher morbidity and mortality among oral cancer patients.

Attributable risk factors are chewing tobacco that comprise of paan, betel quid, betel nut, areca nut, gutka, naswar, cigarette smoking and alcohol consumption. The presence of chronic inflammatory lesions of the oral cavity is also a contributing factor. A dramatic proportion of OSCC develop from pre-malignant lesions such as leukoplakia, *erythroplakia*, oral sub mucous fibrosis and lichen plannus.[7] The current method for diagnosing OSCC involves a thorough oral examination as well as a histological investigation of any suspicious area of the oral cavity. An incisional biopsy collected from the suspected lesion is the gold standard for a conclusive diagnosis of OSCC. There has been a constant search for biomarkers in saliva, a body fluid that can be easily collected, for noninvasive detection of oral cancer and precancerous lesions. Recently developed molecular level technologies such as proteomics, transcriptomics and metabolomics are being explored to help in early diagnosis and the management under the umbrella of PCR. It has been demonstrated that miRNA regulate the drug resistance, apoptosis, and cell proliferation in oral squamous cell carcinomas (OSCCs). The fold/level of expression of miRNA in oral malignancies is different from that of normal tissues.[8] The evidence indicates that changes in the miRNA profile may be identified in saliva that is locally absorbed by malignant cells, and that several techniques may be employed to identify the miRNA secreted in saliva.

MicroRNAs (miRNAs) are a type of short, noncoding RNA molecules that naturally regulate gene expression after transcription, and are known to actively participate in a variety of physiological processes. It has been demonstrated that the pathophysiology of OSCC is linked to dysregulation of microRNA, and providing insight into this dysregulation may inform patient outcomes.[9] Saliva can also contain abnormal genetic material or protein molecules, and analyzing gene expression patterns in saliva can aid in the early detection of cancer. Numerous miRNAs have been discovered and their involvement in various cancers has been confirmed, but the identification and validation of miRNAs in saliva is a relatively new area of study. MiRNAs have distinct expression profiles as they are differentially expressed in cancerous cells when compared to normal cells. Recent research has demonstrated that miR-125a and miR-200a levels are significantly reduced in OSCC, while miR-31 is overexpressed.[10] The detection of oral cancer through salivary biomarkers can provide a non-invasive, cost-effective, and reliable method for the early identification, monitoring, and post-therapy evaluation of oral cancer patients. Significantly effective technique for early evaluation of OSCC are required to improve the management and survival from this fatal disease. Therefore, this research has been planned to provide evidence regarding validity of this test for the early detection of oral cancers.

Methodology

This a descriptive cross-sectional study was conducted in the department of pathology and molecular laboratory, department of oral & maxillofacial surgery of Liaquat University of Medical & Health Sciences, Jamshoro / Hyderabad, from March 2022 to August 2022. The sample size calculation is done using the open Epi software for "Sample size calculation." The prevalence of oral squamous cell carcinoma is 10% keeping this value as a reference value the sample size is calculated by using the following equation and the sample size of the study is then rounded to 120. All histopathologicaly diagnosed OSCC patients of both genders with age group 18 years and above were included. Patients below the age of 18 years, mentally retarded patients, patients with history of immunodeficiency, autoimmune disorders, hepatitis, radiation and if on cytotoxic drugs and those who did not agree to participate in the study were excluded. Non-probability convenience sampling method was used. The permission of Ethical Review Committee of Liaquat University of Medical Health Sciences Hospital was taken

prior to conduct of the study this study was conducted on the study participants who meet inclusion criteria. A written consent was taken from every relevant respondent for participation in the study. For the saliva collection and processing, **w**hole saliva samples (inactivated) were collected from subjects diagnosed as OSCC and controls following standard operating procedures. The subjects were counseled to avoid eating, drinking, smoking or oral procedures for at least 1 h prior to the collection of saliva.

Subjects are asked to rinse their mouth well with distilled drinking water for one minute before taking the saliva samples. After five minutes of oral rinsing spit 5 mL of saliva into a 50 mL sterile tube placed on the ice. The tube should remain on ice while collecting the saliva samples. Four hundred microliters of the whole saliva mixture (200 µL whole saliva and 200 µLRNAlater), and 400 µL of the supernatant saliva was used for RNA extraction. Saliva samples were extracted using the mirVana™ miRNA Isolation Kit according to the manufacturer's guideline. The data was collected on pre designed questionnaire. The data was entered and analyzed by the SPSS version 26.

Results

The study revealed that the mean age of the patients was 47.45 ± 10.85 years, with a range from 30 to 71 years. Among all study subjects, 85 (70.8%) were male, while 35 (29.2%) were female. In terms of marital status, 90.0% were married, 5.0% were unmarried, and an equal proportion were widowed. The majority of cases (70.8%) resided in rural areas compared to 29.2% in urban areas. Additionally, 95.8% of the participants identified as Muslims, with 4.2% being non-Muslims. Occupational status varied, with 40.0% classified as skilled workers, 28.3% as unemployed, 22.5% as unskilled workers, and 9.2% as professionals. Educationally, 35.0% were uneducated, 30.0% had up to intermediate education, 14.2% had secondary level education, 14.2% had primary level education, and only 6.7% were graduates. Out of all, 16.7% cases had positive family history of oral squamous cell carcinoma, and 20.0% cases had comorbidities. The leading causes of oral squamous cell carcinoma were identified as cigarette smoking, Paan (betel quid), chaalia (betel nut), and naswar (smokeless tobacco). Lips, buccal mucosa and tong were the commonest t sites of the oral squamous cell carcinoma. Furthermore, out of all study subjects, most of the cases 59.2% had moderately differentiated SCC, 30.0% cases had well-differentiated SCC and 10.8% of the cases had poor differentiated SCC Table I and II.

Out of all study subjects, 87.5% of the patients had positive micro-RNA expression and 12.5% had negative micro-RNA expression. Figure 1.

Table I: Patients' distribution according demographic and clinical characteristics. (n=120)

Variables		N	%
Gender	Male	85	70.8%
	Female	35	29.2%
	Total	120	100.0%
Marital status	Married	108	90.0%
	Unmarried	06	05.0%
	Widow	06	05.0%
Residential status	Urban	35	29.2%
	Rural	85	70.8%
Religion	Muslim	115	95.8%
	Non-Muslim	5	04.2%
Occupational status	Professional	11	9.2%
	Skilled worker	48	40.0%
	Unskilled worker	27	22.5%
	Unemployed	34	28.3%
Educational status	Graduate	8	6.7%
	Intermediate	36	30.0%
	Secondary	17	14.2%
	Primary	17	14.2%
	Uneducated	42	35.0%
Comorbidities	Yes	24	20%
	No	96	80.0
Family history	Positive	20	16.7%
	Negative	100	83.3%
Biopsy diagnosis	Well, differentiated	36	30.0%
	Moderately differentiated	71	59.2%
	Poor differentiated	13	10.8%

Table II: Risk factors of squamous cell carcinoma. (n=120)

Risk factors	N	%
Unknown	8	6.7
Cigarette smoking only	11	9.2
Cigarette smoking +gutka	7	5.8
Cigarette +Gutka+ Paan+ Chaalia	3	2.5
Cigarette +Gutka+ Paan+ Chaalia +naswar	5	4.2
Cigarette +Gutka+ Chaalia	2	1.7
Cigarette +Gutka+ Paan	6	5.0
Cigarette + Chaalia	13	10.8
Cigarette + naswar	3	2.5
Gutka only	5	4.2
Gutka+ Paan	2	1.7
Gutka+ Paan+ Chaalia	3	2.5
Gutka+ Paan+ Chaalia +naswar	5	4.2
Paan only	12	10.0
Chaalia only	10	8.3
Naswar only	25	20.8
Total	120	100.0

Furthermore, the Micro-RNA expression was significantly associated, with poorly differentiated squamous cell carcinoma (p=0.016). Table III

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Table III: Histological types according to miro-RNA expression. (n=120)

Variables		MicroRNA			p-value
		Positive	Negative	Total	
Biopsy	Well, differentiated	27	9	36	0.016
	Moderately differentiated	22.5%	7.5%	30.0%	
	Poor differentiated	67	4	71	
	Well, differentiated	55.8%	3.3%	59.2%	
	Moderately differentiated	11	2	13	
		9.2%	1.7%	10.8%	
Total		105	15	120	
		87.5%	12.5%	100.0%	

Discussion

One of the most prevalent forms of carcinoma of the head and neck is the OSCC, accounting for approximately 90% of all oral malignancies.[11] Diagnosis of OSCC typically

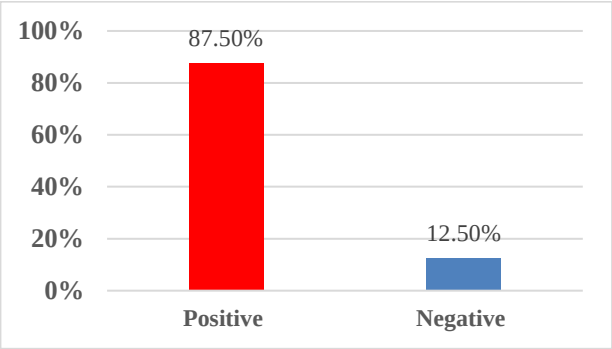

Figure 1. Patients' distribution according to miro-RNA expression. (n=120)

involves a thorough physical examination of the mouth and throat, along with a biopsy of any suspicious tissue. Additional imaging tests, such as X-rays, CT scans, or MRI scans, may be ordered to determine the extent of the cancer and if it has extended to more body parts. This study has been done to evaluate salivary microRNA's diagnostic potential for the early identification of OSCC, contains 120 patients of SCC with an overall average of 47.45±10.85 years and male predominance 70.8%. These findings were supported by the study of Ahmad P et al[12] as large proportion of individuals with cancer of the oral cavity were males accounting for 62.8%, 83.4% without addiction of alcohol, 57.5% nonsmokers, 96.7% non-betel quid chewers, and Malay 68.8% in ethnicity. On the other hand, Nihar RB et al[13] reported that the results of the cross-tabulation of age and gender showed that the majority of patients, 245 (57.24%), were female in the age range of 45-64 years, followed by males in the same age group with 480 (51.39%) patients.

Consistently Anwar N et al[14] revealed that a total of 186 individuals were sampled, with 149 males and 37 females (4:1 ratio), along with the mean age of the patients was 47.6 years. Alamgir MM et al[15] also found comparable findings as patients average age was 47.1± 12.22 years and in their study, there were 52 female patients as well as 98 male patients. The male predominance in OSCC may be due to several factors. Firstly, higher rates of tobacco and alcohol consumption among males, both of which are significant risk factors for oral cancer, contribute to this trend. Additionally, men are more often exposed to carcinogens in certain industries due to their higher representation, further increasing their risk and some biological factors such as hormonal differences and genetic predispositions may also play a role.

In this study the cigarette smoking, pan, chaalia and naswar were the commonest cause of the oral squamous cell carcinoma. Similarly, Anwar N et al[14] stated that just 22% of the cases in their study being regular smokers, and that there was no correlation between smoking and chewing behaviors, site of the tumor, or tumor progression.[16] As a five-year predictor of secondary primary tumors of OCSCC, it was not individually predictively significant, despite its proven association with HNSCC. Smoking has been found in numerous studies to have an adverse synergistic effect on chewing and consumption of alcohol, with the oropharynx or larynx being the subsites most frequently affected rather than the cavity in the mouth.[17] In aligns to this study Tenore G et al[18] observed that the consuming alcohol and smoking have been linked to a higher risk of oral squamous cell cancer. Cigarette smoking contains numerous carcinogenic chemicals that can damage the DNA in the cells lining the oral cavity, leading to the development of cancerous tumors. It is estimated that smokers are six times more likely to develop OSCC than non-smokers.

Additionally, the use of pan, chaalia, and naswar can also cause chronic irritation and inflammation of the oral tissues, which can increase the risk of developing cancer. Furthermore, these products often contain other harmful additives, such as lime, spices, and areca nut, which can further increase the risk of developing cancer.

In this study out of all study subjects, 87.5% of the patients had positive micro-RNA expression and micro-RNA expression was significantly associated, with poorly differentiated squamous cell carcinoma (p=0.016). Consistently Manikandan M et al[19] demonstrated that the identification of the disrupted signaling pathways and differently expressed miRNAs in OSCC has consequences for the creation of new treatment approaches. Our findings were also in closed with the study by Gissi DB et al[20] and Rajan C et al[21] further noted that in their investigation, a miRNA characteristic for OSCC with predictive value and that the expression ratio of mir-196a/miR-204 revealed as the greatest predictor for recurrence of disease and the survival of patients. However, this study has shown that microRNAs (miRNAs) could potentially be used as non-invasive biomarkers for the detection of OSCC. MiRNAs are small, non-coding RNA molecules that regulate gene expression by binding to specific messenger RNAs (mRNAs). Dysregulation of miRNAs has been linked to various diseases, including cancer. Certain miRNAs have been demonstrated shown to be either raise- or decreased in OSCC, dependent on the severity and stages of the disease. MiRNAs have been investigated in a number of studies as possible biomarkers for diagnosis of OSCC.[22-25]

According to these findings, individuals who have OSCC can be distinguished from healthy persons or sufferers with benign oral lesions based on the miRNA expression patterns found in their saliva, blood, and tissues specimens. Additionally, miRNAs can forecast an OSCC the individual's prognosis and responsiveness to treatment. Compared to conventional diagnostic techniques like biopsy and image processing, non-invasive OSCC detection with miRNAs offers a number of benefits. It is more rapid, less invasive, and may be able to identify OSCC early on, when it is easier to treat. Furthermore, miRNA-based diagnostics have the potential to be used for OSCC diagnosis and therapy because they are easily standardized and applied in clinical settings. However, before widespread clinical usage, a number of limitations need to be addressed. Age, gender, and exposure to various settings can all affect the expression of miRNA. This diversity can make the development of consistent and reliable miRNA-based diagnostic tests for OSCC difficult.

There isn't a defined process in place yet for collecting and analyzing miRNA samples. The absence of standardization can lead to a range of outcomes, making it difficult to compare and assess research findings. We must evaluate these tests' cost-effectiveness to ensure that they are practical and beneficial for routine clinical use. More research is advised to validate the findings and establish standardized protocols for collecting and conducting an analysis of miRNA samples.

Conclusion

Study revealed that patients suffering from OSCC exhibited significantly elevated levels of salivary miroRNA expression. Conversely, the use of salivary biomarkers such as miRNAs allows for the identification and tracking of OSCC across various histological grades. Timely detection and intervention have the potential to diminish OSCC-associated morbidity and mortality rates, ultimately enhancing survival outcomes. Consequently, salivary biomarkers are recognized as a valuable asset in the management of OSCC.

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