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Exploiting the miRNA-21 Biomarker in Tonsil Squamous Cell Carcinoma

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Abstract

Purpose: The rate of tonsil oropharyngeal cancers is increasing. MicroRNAs (miRNAs) are identified as small, noncoding, endogenously expressed RNAs that target mRNAs and regulate the post-transcriptional levels of protein expression. MiRNA-21(miR-21) is significantly increased in patients with oral carcinoma and has oncogenic roles in oral carcinogenesis. Therefore, miRNAs have been recommended to be used in the early diagnosis of HNC.
Methods - rt-qPCR was utilized to evaluate the expression levels of human salivary miR-21. Samples were collected from individuals diagnosed with tonsil squamous cell carcinoma (TSCC) (n = 45) in both whole saliva and supernatant saliva. Then these levels were compared to those of a control cohort consisting of healthy individuals (n = 45) to determine differential expression patterns.
Results- Salivary miR-21 displayed a notable elevation in all stages of tonsil squamous cell carcinoma (TSCC), including cases involving small tumors. The initial analysis revealed a significant up-regulation of both whole saliva and salivary miR-21 of the supernatant in patients with TSCC compared to controls (P= 0.01, 0.02 respectively). Furthermore, miR-21 showed a higher abundance in whole saliva in contrast to its levels in supernatant saliva. These observations confirm the potential of salivary miR-21 for the detection of tonsil malignancies.
Conclusions This investigation underscores the ability of salivary miR-21 for the detection and ongoing prognostic monitoring of tonsil squamous cell carcinoma (TSCC), both in whole saliva and in supernatant saliva samples.

Keywords: *miR-21, whole saliva, supernatant saliva, tonsil cancer.*

Introduction

Head and Neck Cancer (HNC) represents a significant contributor to global cancer-related mortality, highlighting its substantial impact (1). Australia witnessed a marked rise in HNC cases from 1986 to 2006 (2). The steady annual increase in oropharyngeal carcinoma rates, particularly in Queensland and New Zealand (at 10.6% and 11.9% respectively) since 2005, underscores the need for deep investigation (3). Although human papillomavirus remains a recognized risk factor for tonsil squamous cell carcinoma (TSCC), the research aims to elucidate the molecular pathways that influence its prognosis (4). In particular, established factors such as alcohol and smoking contribute significantly to oropharyngeal carcinoma (5). The delayed diagnosis of Squamous Cell Carcinoma (SCC) in advanced stages significantly affects mortality rates and prognostic outcomes (6), often due to increased lymph node metastases. This underscores the need for novel biomarkers that allow early diagnosis and prognostic assessment in oropharyngeal carcinoma. MicroRNA (miRNA), a small noncoding RNA, intricately regulates mRNA to modulate protein production and plays a role in the progression of oncogenesis (7). Multiple studies emphasize the substantive role of oncogenic miRNAs, particularly the increased presence of miR-21 in oral cancer (8). This study explores the potential for elevated miR-21 levels early diagnosis and postoperative prognostication in TSCC. The findings highlight an up-regulation of salivary miR-21 in both whole saliva and supernatant saliva samples (supernatant saliva is more concentrated than whole saliva and gives more purification RNA) among patients with TSCC, pointing to its viability as a diagnostic biomarker for oropharyngeal carci-

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noma.

Methods

Patients

This investigation obtained ethical clearance from the Queensland Government. Saliva samples were obtained from 45 individuals diagnosed with TSCC at Gold Cost Hospital in bilateral sites, different ages and sex, and oral habits (smoking and alcohol) matched with healthy counterparts, before surgery. Comprehensive consent was obtained from the patients. Samples were collected between December 1, 2015, and July 30, 2016.

The saliva samples collected from patients diagnosed with Tonsil squamous cell carcinoma (TSCC). Detailed clinical parameters of the subjects are delineated in Table 1.

We probably used a comparative approach, juxtaposing clinical characteristics between individuals diagnosed with TSCC and their healthy counterparts to examine potential differences. Further specific clinical data and insights are shown in Table 1.

Table1. Clinical parameters of subjects

	TSCC	Control	P value
Age, mean	51.7	52.7	(ns) 0.36
Sex, male/female	27/18	24/21	(ns) 0.99
Malignancy History	0	0	
Oral habits			
Alcoholic consumption	21	15	(ns) 0.9
Cigarette smoking	18	15	(ns) 0.9
Site			
Left Tonsil SCC	18		
Right Tonsil SCC	27		
T/N/M classification			
T1	6		
T2	9		
T3	15		
T4	15		
N0	0		
+N	45		

Saliva Collection

Samples were obtained during the morning hours, following established procedures as mentioned in (10, 11). Before collection, individuals were instructed to fast. A mouth rinse with water was recommended to minimize sample contamination. A five-minute interval was observed before participants were seated upright to expectorate saliva in a 50 mL RNA / DNA tube, maintained at a low temperature on ice.

Total RNA Extraction

Total RNA within the range of 1050- ng was reverse-transcribed to complementary DNA (cDNA) using the miRNA reverse transcription kit (Origene, HP 100042). Each 10-µl reaction mixture consisted of 1 to 2 µg of RNA, 1 µl of poly A tailing buffer, 1 µl of mM ATP equal to 0.0001 molar), 1 µl of polymerase (A) and adjustment to 10 µl using nuclease-free water. Polyadenylation was maintained at 37 ° C for two hours.

Next, 1µLof oligo dT primer was introduced into the reaction and incubation at 70˚C for five minutes, followed by immediate cooling on ice for 2 minutes. For the final cDNA synthesis, 4 µl of 5X MMLV buffer was added, incubated at 42˚C for 1 hour, denatured at 95˚C for 5 minutes, and promptly cooled on ice. The resulting reaction mixture was diluted with 200 µL molecular water and stored at -20 C for subsequent use in the quantitative real-time polymerase chain reaction (RT-qPCR).

Real-Time Quantitative Polymerase Chain Reaction

To determine the validity of miRNA candidates previously identified, RT-qPCR assays were performed using the SYBR Green assay (Bio-Rad, HP 1725017-). Specific forward primers and a universal reverse primer, following the manufacturer›s guidelines (Origene), were applied

for miRNA detection, detailed in Table 2. SYBR Green fluorescence-monitored PCR amplicons. Each reaction was carried out in triplicate, following the cycling conditions: initial denaturation at 95 C for 30 seconds, followed by 42 cycles of 95 C for 15 seconds, 55 C for 10 seconds and 72 C for 30 seconds.

Subsequently, melt curve analysis ensured the specificity of the PCR products, varying from 60.0 to 95.0˚C at 0.5˚C increments of 0.5 C for 0.05 minutes. All cycle threshold (Ct) values were below 35. A no template control (NTC) served as the negative control. Using a Quant Studio 6 flex system (Applied Biosystems), RT-qPCR reactions were performed, and Δct values were calculated, normalized against the housekeeping miRNA-16.

Table 2. Primers and probes used for miRNA-specific RT-qPCR.

Name	Forward primer	Reverse primer
miRNA-21	GCTTATCAGACTGATGTTG	GAACATGTCTGCGTATCTC
miRNA-16	AGCAGCACGTAAATATTGG	GAACATGTCTGCGTATCTC

Statistical analyses

The samples were classified into two distinct groups: normal and progressive TSCC. Quantification of miRNA expression levels involved computing the normalized threshold cycle number Δct, calculated as Δct = [Ct (Target miRNA)] - [Ct (miR-16)]. Relative expression levels were determined using the formula 2^-(Δct), a standard method in miRNA genome-wide profiling studies.

Data analyzes were executed using Prism software, version 6. Statistical significance was determined by a threshold p-value of less than 0.05, indicating significance in the findings.

Results

Forty-five saliva samples from Gold Coast University Hospital patients with TSCC and 45 from healthy adults were compared based on tumor characteristics, demographics, and histopathological characteristics (Table 1). The mean age for patients with TSCC was 51.7 ± SD years and for healthy individuals it was 52.7 ± SD years (P = 0.36, Table 1). The gender distribution and habits such as smoking and alcohol consumption did not show significant differences between the two groups (P = 0.9 for all).

MiR-21 was investigated in the saliva of the whole and supernatant of patients with TSCC using RT-qPCR. While miR-21 levels were comparable between whole and supernatant saliva ((supernatant saliva is more concentrated than whole saliva and gives more purification RNA) in patients with TSCC (P = 0.07, Figure 1), its expression was significantly elevated compared to healthy controls (P = 0.01, 0.02 for whole and supernatant saliva, respectively, Figure 2).

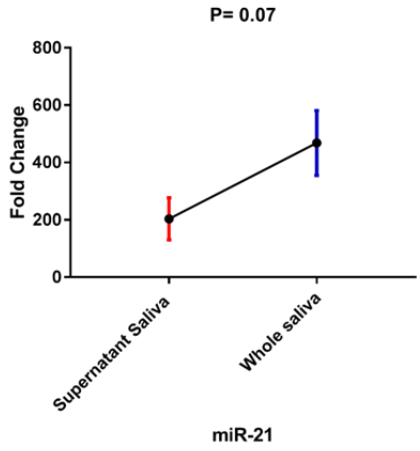

Figure 1: Correlation of miRNA21 (miR-21) levels between whole saliva and supernatant saliva

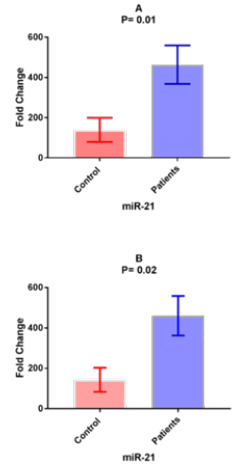

Figure 2: The expression level of salivary miR21 in patients with tonsil SCC normalized to expression in adjacent healthy individuals (15 patients and 15 healthy individuals were analyzed in this sectional study). An expression level of miR-21 in whole saliva. B Expression level of miR-21 in saliva from the supernatant

Regarding clinical factors, miR-21 expression in supernatant saliva remained unaffected by gender, age, or alcohol status (P = 0.189, 0.913, and 0.6008 respectively, Table 3). However, smokers exhibited a markedly higher expression of miR-21 (P = 0.017, Table 3).

Table 3. Relationships between miR-21 expression levels (means ± SEM) and clinical characteristics of TSCC patients

Feature	n	Means ± SEM	*P value
Gender			(ns) 0.189
Male	**27**	1.844 ± 17.1	
Female	**18**	5.995 ± 39.9	
Age			(ns) 0.913
55 ≤	**18**	4.505 ± 25.5	
55>	**27**	2.245 ± 27.3	
Alcoholic			(ns) 0.6008
Yes	**21**	3.880 ± 23.1	
No	**24**	N=3 1.534 ± 33.3	
Smoking			(s) 0.0179
Yes	**18**	0.7576 ± 11.7	
No	**27**	5.014 ± 48.09	

*Student t-test

The study suggests the potential for the detection of miR-21 in saliva samples from TSCC patients, similar to previous tissue-based research (9, 12). Additionally, analysis of miR-21 expression in different stages of TSCC revealed significant differences between various clinical subsets (P = 0.04, 0.004 for different stages of the disease, Figure 3).

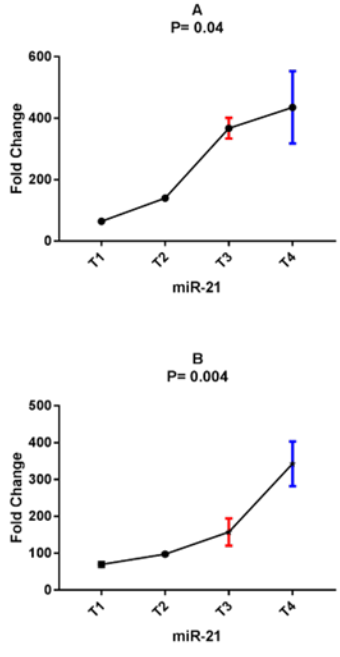

Figure 3: Expression of miR21 in different tumor stages. A. supernatant saliva, B whole saliva

Discussion

The planning of treatments and the management of recovery for patients with head and neck cancer (HNC) present considerable complexities. Identifying specific biomarkers, notably in saliva, holds crucial importance for the early detection, ongoing monitoring, and prognosis of Tongue Squamous Cell Carcinoma patients. Saliva, which encompasses tonsil tissue, offers a non-invasive sampling avenue for potential biomarkers (13, 14). The aim of our study was to determine miR-21 expression in tonsillar tumor. The results of miRNA expression were then compared in whole saliva samples and supernatant saliva samples.

Although mechanistic studies exploring the roles of microRNAs in tumorigenesis are progressing rapidly, translational research using microRNAs as biomarkers is still in an early stage. However, microRNAs exhibit remarkable stability in cells, tissue samples (whether archived or fresh), and various biofluids. This stability makes microRNA-based biomarkers less susceptible to minor variations in sample processing, offering a significant advantage over other types of biomarkers.

MicroRNAs (miRNAs), small noncoding RNA molecules of around 22 nucleotides, play pivotal roles in RNA silencing and gene expression regulation. They can act as tumor suppressors or oncogenes (15) and intricately control almost

30% of human genes, affecting the balance between oncogenes and suppressor genes (16-18). This study identified aberrant expression of salivary miR-21 in patients with TSCC compared to healthy controls. miR-21 is one of the earliest identified cancer-promoting 'oncomiRs', targeting numerous tumor suppressor genes involved in proliferation, apoptosis, and invasion (17). The regulation of miR-21 and its role in carcinogenesis have been studied (17). Recent research has focused on its diagnostic potential. Further investigation of miR-21 as a biomarker and target for cancer treatments is likely to improve the outcomes of cancer patients. This study agrees with others in identifying miR-21 as the miRNA most commonly elevated in cancers. The present findings highlight the importance of miR-21 in the diagnosis of malignancies.

Salivary miRNAs exhibit robust structural stability, resisting harsh conditions such as freeze-thaw cycles, extreme pH levels, prolonged storage, and boiling (14, 19). Consequently, miRNAs are promising candidates as cancer biomarkers in saliva (19-26).

Our current study confirms the feasibility of using microRNAs from an archived saliva sample as biomarkers to discriminate TSCC from non-cancerous control tissues.

Salivary miRNAs originate from various sources, including blood cells, oral epithelial tissues (such as salivary glands, mucosa and gingival pockets), and systemic circulation (14, 27). The composition of saliva reflects oral health, systemic conditions, and general health status. Among these, miRNA-21 consistently exhibits increased expression in various cancers, particularly head and neck cancer, establishing itself as a crucial clinical biomarker and a potential target for therapeutic interventions in oral cancer (9). miRNA-21 interacts with multiple genes, including programmed cell death4 (PDCD4) and phosphatide and tensin homolog (PTEN) on chromosome 10, influencing tumor suppression mechanisms (12, 28, 29). Its involvement in the Ras phosphoinositide-3-kinase (PI-3K) pathway underscores its role in modulating tumor suppressor genes and apoptosis (12, 30). Moreover, a recent study revealed that decreased regulation of miR-21 may effectively reverse drug resistance in many types of cancer (31).

Conclusions

In summary, this study employed a cross-sectional approach to characterize miR-21 expression in tonsillar cancers. Saliva is being explored as a promising sample for disease detection because of its convenience, noninvasiveness, and cost-effectiveness compared to blood collection. Our investigation specifically focused on gauging the levels of miR-21 in saliva and assessing its potential as a discriminatory marker in Tongue Squamous Cell Carcinoma. The findings suggest that salivary miR-21 holds promise as a valuable biomarker for TSCC. More research is advised to deepen our understanding of the significant relationship between miR-21 and TSCC. Importantly, our observations reveal that salivary miR-21 expression levels remained consistent regardless of staging or nodal metastasis. Previous studies have also underscored the presence of miR-21 in various head and neck cancer specimens. Consequently, altered expression of miR-21 in saliva can indicate an early molecular occurrence in the pathogenesis of TSCC, demonstrating limited variability among patients with TSCC. The increased expression of salivary miR-21 emphasizes its potential as an effective biomarker for early detection of TSCC. In conclusion, further scientific exploration of the role of salivary miR-21 in TSCC is warranted and essential.

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Conflict of interest: The authors declare that they have no conflict of interest.

Authors contribution: All authors participated in the development of the search strategy, study design, quality assessment, and analytical strategy for this study. Rushdi Fadhil participated in sample collection and laboratory technique. Rushdi Fadhil, Raj Nair and Ming Wei participated in statistical analysis, data interpretation, and critically reviewed the manuscript.

All authors read and approved the final version of the manuscript.

Ethical approval: the work approved by the scientific committee of the Iraqi Center for Cancer and Medical Genetic Research, Baghdad, Iraq, No. 738 dated: 29-01-2021.

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