10.21608/adjc.2020.36679.1074 Advanced Dental Journal
Volume 2 (2020) | Issue 4| Pages 209-217

Original Article

miR-21 and TAC as Salivary Biomarkers for Oral Dysplasia

Shereen Ali[1], Maha Abdelkawy[2], Sherif Ali[3], Laila Rashed[4]

[1]Oral Medicine and Periodontology, Faculty of Dentistry, Cairo University, Cairo, Egypt.
[2]Oral Medicine and Periodontology, Faculty of Dentistry, Beni-Suef University, Beni-Suef, Egypt.
[3]Oral and Maxillofacial Surgery, Faculty of Dentistry, Cairo University, Cairo, Egypt.
[4]Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt.

Email: cherriesah@hotmail.com

Submitted: 21-7-2020
Accepted: 16-10-2020

Abstract

Objectives: Previous studies have demonstrated that microRNA-21 (miR-21) and total antioxidant capacity (TAC) could be potential diagnostic biomarkers for oral squamous cell carcinoma. Their diagnostic potential in the early stages of carcinogenesis is not clear yet. This study was conducted to determine the salivary levels of miR-21 and TAC in patients with oral hyperplasia and dysplasia.
Methods: We assessed expression of miR-21 and TAC in whole unstimulated saliva samples of 30 patients with oral mucosal lesions demonstrating hyperplastic or dysplastic changes and 30 healthy individuals with normal mucosa. Biopsy was taken from the lesions for histopathologic assessment. Statistical analysis was performed with IBM® SPSS® ($P \leq 0.05$) and ROC curve analysis was conducted with MedCalc.
Results: miR-21 expression varied among the studied groups with significant difference. However, TAC expression varied only between mucosal lesions and normal mucosa with significant difference. Diagnostic accuracy, sensitivity, specificity, positive predictive value and negative predictive value were higher in miR-21 than TAC.
Conclusions: Salivary miR-21 are more accurate in detecting oral dysplasia than salivary TAC. Salivary miR-21 could be potential diagnostic biomarker for screening and early detection of oral cancer. More studies are required to validate miR-21.

Keywords: miRNA, total antioxidant capacity, dysplasia, oral cancer, biomarkers

Introduction

Oral cancer is a major oral health problem, its early detection remains a global burden that challenges oral medicine researchers and specialists. The world health organization confirmed that early diagnosis is the most important goal to combat oral cancer because it can reduce predicted cases, enable more effective management, allow selection of conservative therapeutic approaches, improve quality of life, increase chances of favorable prognosis and reduce morbidity rate [1-3].

Clinical examination augmented by histopathological assessment remain the gold standard for detection of oral cancer. However, their diagnostic power is restricted in the early

stages of carcinogenesis and their prognostic value is limited in stratifying oral mucosal lesions as "progressive" and "non-progressive". Many tests are being developed to complement clinical examination and facilitate early cancer detection [1, 4-6].

Both the last Cochrane review and the American dental association (ADA) recommendations agreed that none of the adjunctive tests can be recommended as a replacement for the gold standard biopsy and histopathological assessment [3, 7]. However, the Cochrane review encouraged researchers to explore the diagnostic potential of salivary biomarkers [3]. Moreover, the ADA recommended that the major indication for salivary adjuncts could be for individuals with no lesions, early stage lesions, or non-classical lesions if their diagnostic accuracy is improved [7].

Saliva is one of the most recommended detection media, it provides relevant information regarding oral lesions due to its direct contact with oral lesions. Saliva has various pros such as easy accessibility, non-invasiveness, patient comfort, safe handling with low chances of disease transmission, convenience for multisampling and requiring minimal training. However, we should be aware of the cons including lack of standardized procedures, variability in the levels of the salivary markers and validation in inflammatory conditions [8,9].

In the current study, we have selected two attractive families of salivary biomarkers; the classical oxidants-antioxidants and the emerging microRNAs (miRNAs). Observations suggested that oxidative stress is involved in carcinogenesis and play role in oral cancer susceptibility. Researchers associated low levels of antioxidants with increased oral cancer risk. Total antioxidant capacity (TAC) is a composite biomarker for the combined effects of individual antioxidants [10, 11].

Regarding miRNAs, they are proposed to be the third diagnostic salivary biomarkers beside proteomes and transcriptomes. miRNAs function in concert with tumor suppressors & oncoproteins to regulate key cellular events including development, differentiation and carcinogenesis. microRNA-21 (miR-21) is a well-known oncogenic miRNA that is overexpressed in many carcinomas, solid and hematological tumors [4, 12, 13].

Previous studies have demonstrated that miR-21 and TAC could be potential diagnostic biomarkers for oral squamous cell carcinoma (OSCC). However, their diagnostic potential in the early stages of carcinogenesis is not clear yet. The current study was conducted to 1) determine the salivary levels of miR-21 and TAC in patients with oral hyperplasia and dysplasia and 2) assess and compare the diagnostic potential of salivary miR-21 and TAC in patients with oral hyperplasia and dysplasia.

Materials and Methods

Participants and Histopathological evaluation:

We assessed salivary expression of miR-21 and TAC in 30 patients with oral mucosal lesions demonstrating hyperplastic or dysplastic changes and 30 healthy individuals with normal mucosa. Participants were recruited from the pool of the clinics of Oral Medicine & Periodontology Department and Diagnostic center, Faculty of Dentistry, Cairo University, Egypt.

This study followed the principles of the Helsinki Declaration and was approved by the Research ethics committee of Faculty of Dentistry, Cairo University, Egypt (Code: 16924). Each participant was informed about the details of the study and signed a written consent.

Participants were enrolled in a consecutive order after confirming that they were eligible for the study. Individuals who agreed to sign the informed consent were included. Exclusion criteria were medications, antioxidant vitamins or other supplements; periodontal diseases; local infections; systemic diseases; systemic infections; smoking and xerostomia.

A double wedge incisional biopsy was taken from the oral mucosal lesions. Biopsy material was immediately fixed in formalin, then processed for preparing a paraffin block. Five µm tissue sections were then cut, stained with

conventional Hematoxyline & Eosin (H&E) for histopathologic assessment.

Salivary sample collection:

A whole unstimulated saliva sample was collected from each participant in the morning. The participants were asked to refrain from eating, drinking, brushing their teeth, using mouth rinse, or chewing gum at least two hours prior to sample collection. Each participant was asked to swallow, then tilt the head forward, and expectorate unstimulated whole saliva into a sterile tube. The collected material was stored at -80 °C, until analysis. Saliva sample was collected before performing the tissue biopsy.

Analysis of saliva:

Detection of salivary TAC:

Salivary TAC was measured on an Olympus AU-600 analyzer using the TAC kit (Medikon SA, Gerakas, Greece). Antioxidants in the sample inhibited bleaching of crocin from ABAP [2, 2-azobis-(2-amidinopropane) dihydrochloride] to a degree that is proportional to their concentration. The assay was performed at 37°C as follows: 2 µl of sample was mixed with 250 µl of crocin reagent and incubated for 160 seconds. Subsequently, 250 µl of ABAP were added and the decrease in absorbance at 450 nm was measured 26 seconds later.

Detection of salivary miR-21:

The saliva samples were vortexed then centrifuged at 2,600 × g for 15 minutes at 4°C. The saliva supernatant was collected, for every milliliter 1 µL (20 U) of SUPERase inhibitor was added and kept frozen at -80°C.

Total RNA was isolated from saliva supernatant using mirVana miRNA isolation Kit (Applied Biosystems). RNA concentrations were measured by spectrophotometer. cDNA was generated using Prime Script RT reagent kit (Applied Biosystem) in a 20 µl final reaction volume containing 0.5 µg of RNA, 0.5 µl Prime-Script RT enzyme mix, 4 µl 5×PrimeScript buffer and 1 µl RT primer, and incubated at 42°C for 60 minutes, then at 85°C for 5 minutes.

Quantitative real-time PCR assay was performed to evaluate miR-21 expression using SYBR Premix Ex Taq (Applied BioSystem). Primers for miR-21 and endogenous control U6 snRNA were obtained from Applied Biosystems and measured in Step One Plus System supplied by (Applied BioSystem, USA). The cycling was denaturing at 95°C for 10 minutes, followed by 45 cycles of denaturation (95°C - 15 seconds), annealing (60°C - 30 seconds) and extension (72°C - 1 minute). The relative expression of miR-21 was calculated and normalized using the 2-ΔΔCt method relative to U6 small nuclear RNA.

Statistical analysis:

The mean and standard deviation values were calculated for each group in each test. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests, data showed parametric (normal) distribution. Comparisons between the two studied groups was done by Student-t-test. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows. A receiver operating characteristic (ROC) curve was created for each biomarker to evaluate its diagnostic performance and area under the ROC curve (AUC) was calculated. ROC curve analysis was conducted with MedCalc.

Results

Demographic data:

The normal mucosa group included 30 individuals; 7 females and 23 males. Their age ranged from 19 to 61 years old with mean 40.8. The oral mucosal lesions group included 30 patients; 5 females and 25 males. Their age ranged from 20 to 63 years old with mean 40.4. The oral mucosal lesions were 12 oral keratosis and 18 leukoplakia.

The patients suffering from oral mucosa lesions with hyperplasia were 2 females and 13 males, their age range was (20-60) years old with mean 38.4. Distribution of lesions was 8 oral keratosis and 7 leukoplakia. While, patients suffering from lesions with dysplasia were 3 females and 12 males, their age range was (29-63) years old

with mean 43.4. The oral mucosal lesions were 4 oral keratosis and 11 leukoplakia.

TAC and miR-21 expression:

TAC expression ranged from 17.5 to 40.8 µmol/l in the normal mucosa, 9.5 to 38.7 µmol/l in the mucosal lesions, 10.2 to 38.7 µmol/l in hyperplasia and 9.5 to 37.3 µmol/l in dysplasia. The lowest mean value was detected in dysplasia, followed by mucosal lesions, then hyperplasia and the highest mean value was scored in normal mucosa.

TAC expression was lower in mucosal lesions than normal mucosa with significant difference (P = 0.001), and in dysplasia than hyperplasia but with no significant difference (P = 0.093) (Table 1 - Figure 1).

miR-21 expression ranged from 0.79 to 2.1 in the normal mucosa, 2.1 to 16.2 in the mucosal lesions, 2.1 to 10.6 in hyperplasia and 10.2 to 16.2 in dysplasia. The highest mean value was detected in dysplasia, followed by mucosal lesions, then hyperplasia and the lowest mean value was scored in normal mucosa.

miR-21 expression was higher in mucosal lesions than normal mucosa and in dysplasia than hyperplasia with significant difference (P < 0.001) (Table 1 - Figure 2).

Potential diagnostic power of TAC and miR-21:

AUC was high in miR-21 reaching up to 0.986, while in TAC it was only 0.671. Diagnostic accuracy, sensitivity and specificity were higher in miR-21 (96.6%, 100%, 94.4%, respectively) than TAC (73.3%, 83.3%, 66.7%, respectively). Positive predictive value and negative predictive value were higher in miR-21 (92.3%, 100%, respectively) than TAC (62.5%, 85.7%, respectively). The cutoff value was 9.45 for miR-21 and 20.85 for TAC (Table 2 – Figures 3 & 4).

Discussion

Development of salivary biomarkers is essential for early detection of oral cancer [7, 14]. Up to our knowledge, this is the first study to compare the diagnostic potential of members from two important families in early stages of carcinogenesis; namely miR-21 and TAC in patients with oral hyperplasia and dysplasia. In addition, it is the first study to assess the diagnostic accuracy of TAC in patients with oral hyperplasia and dysplasia.

The total protective capacity of antioxidants against oral cancer is achieved by balance and interaction of all antioxidant components, accordingly a composite marker should be the target marker. TAC is a composite biomarker that can provide insight into the overall oxidant-antioxidant balance. Moreover, it is valuable for monitoring changes in endogenous antioxidant levels [10].

The oncogenic, miR-21, might have a pivotal role in carcinogenesis and invasiveness. It promotes cell proliferation and inhibits apoptosis through regulating target molecules and pathways as tropomyosin I (TPM1), programmed cell death 4 (PDCD4) and phosphatase, tensin homolog (PTEN) & Wnt/□-catenin pathway [4, 13].

In the current study, miR-21 expression was higher in oral mucosal lesions than normal mucosa with significant difference. Our results are in accordance with Zahran et al. and Hung et al. who reported that salivary miR-21 expression is significantly increased in potentially malignant lesions compared to controls [15, 16]. The current study results are consistent with Cervigne et al. and Brito et al. [4, 13]. Cervigne et al. documented that tissue miR-21 was detected in progressive leukoplakia, but not in normal oral mucosa [4] and Brito et al. found that tissue miR-21 expression is significantly increased in oral leukoplakia compared to normal oral mucosa [13].

In addition, our results revealed that miR-21 expression that was higher oral mucosal lesions with dysplasia than lesions with hyperplasia with significant difference. This agrees with Zahran et al. who detected significantly higher values for salivary miRNA-21 in potentially malignant lesions with dysplasia compared to those without dysplasia.

Table 1: Salivary TAC and miR-21 expression in the studied groups

	Normal mucosa (n= 30)	Oral mucosal lesions (n= 30)	P value	Hyperplasia (n= 15)	Dysplasia (n= 15)	P value
TAC	29.36 ± 6.59	21.56 ± 9.99	<0.001*	24.38 ± 9.9	18.00 ± 8.97	0.093
miR-21	1.18 ± 0.35	8.85 ± 4.39	<0.001*	5.81 ± 2.42	13.15 ± 1.87	<0.001*

*: significant ($p < 0.05$)

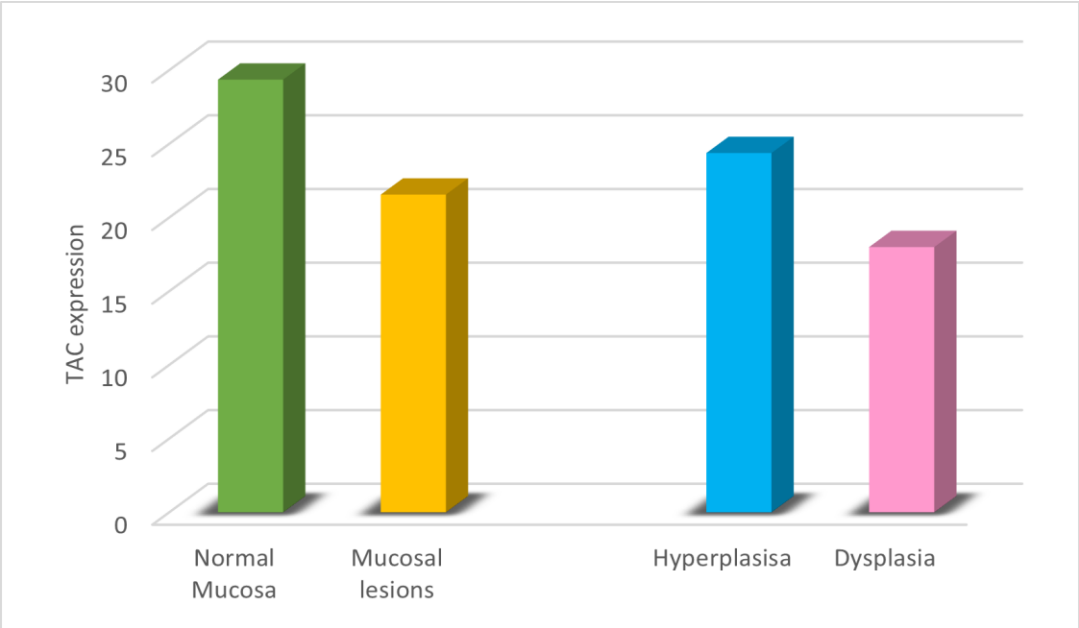

Figure 1 Comparison of salivary TAC expression among the studied groups

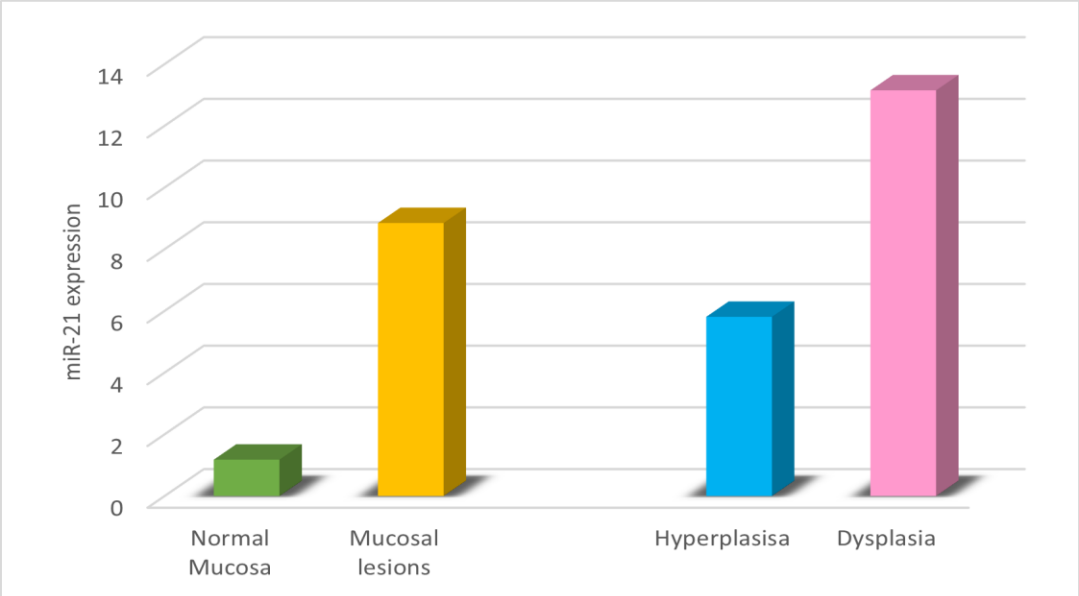

Figure 2 Comparison of salivary miR-21 expression among the studied groups

Table 2: ROC curve analysis of TAC and miR-21

	Cut-off	Sensitivity (%)	Specificity (%)	+PV (%)	-PV (%)	Diagnostic accuracy (%)	AUC
TAC	20.85	83.3	66.7	62.5	85.7	73.3	0.671
miR-21	9.45	100	94.4	92.3	100	96.6	0.986

+PV: Positive predictive value; -PV: Negative predictive value

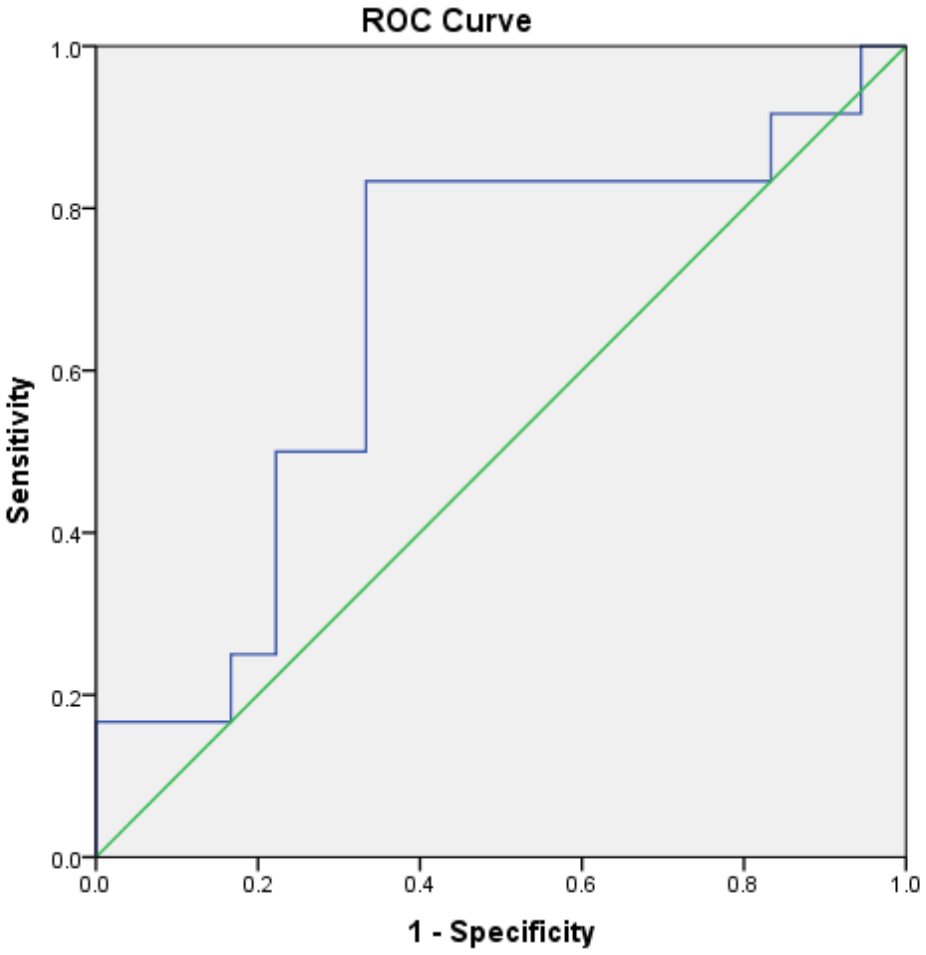

Figure 3 ROC curve demonstrating the diagnostic accuracy of salivary TAC

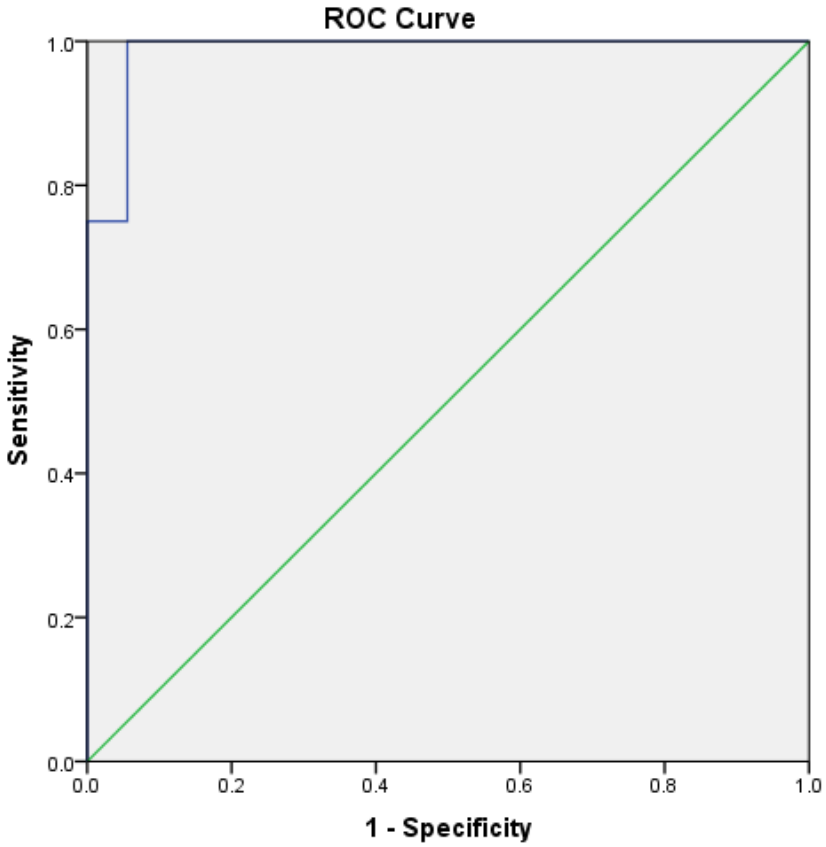

Figure 4 ROC curve demonstrating the diagnostic accuracy of salivary miR-21

Our results are in harmony with Brito et al. who reported that oral leukoplakia with dysplastic changes showed higher tissue miR-21 expression than leukoplakia without dysplasia [15]. Cervigne et al. confirmed consistent increase in tissue miR-21 expression in association with increased lesion severity and reported overexpression of miR-21 in progressive dysplasia, but not in non-progressive dysplasia [4].

Regarding TAC expressions, it was lower in mucosal lesions than normal mucosa with significant difference, but in dysplasia than hyperplasia with no significant difference. All previous studies confirmed that TAC expression was lower in potentially malignant lesions compared to normal mucosa [10, 11, 17, 18]. Korde et al. and Vlkova et al. reported that the difference is statistically significant, serum or salivary expression respectively [10, 11]. On the other hand, Agha-Hosseini et al. and Babiuch et al. revealed that the difference of the salivary expression is statistically insignificant [17, 18]. These studies result together with our result revealed that whenever we narrow our scope to a nearby successive steps of carcinogenesis the value of TAC decrease.

The limited power of TAC in closely successive steps was highlighted in other studies but in advanced stages of carcinogenesis. Shetty et al. found that pre- and post-operative serum TAC in oral cancer patients were significantly lower than normal value, however there was no statistically significant difference between pre- and postoperative levels [19]. Pakfetrat et al. stated that there was no significant relationship between the level of serum TAC, and the stage or grade of head and neck squamous cell carcinoma [20].

Regarding the diagnostic performance of miR-21, the current study demonstrated high

diagnostic accuracy, sensitivity and specificity (96.6%, 100%, 94.4%; respectively). A previous study that evaluated the diagnostic performance of miR-21 in potentially malignant lesions and oral cancer documented sensitivity and specificity range (65-90%, 60-65%; respectively) [15].

In the present study, the diagnostic performance of TAC was low. Diagnostic accuracy, sensitivity and specificity were 73.3%, 83.3% and 66.7%, respectively. No previous studies tested the diagnostic performance of TAC.

ROC curve analysis of the current study revealed that diagnostic accuracy, sensitivity, specificity, positive predictive value and negative predictive value were higher in miR-21 than TAC. AUC was high in miR-21, while in TAC it was low. These results suggest the utility of miR-21 as a potential diagnostic biomarker for detection of dysplasia.

The limitations of the current study include the relatively strict eligibility criteria which could affect the generalizability of the results. Being the first study to compare the two biomarkers in this population, we thought to provide insights of the selected biomarkers without the effect of confounders accordingly we excluded them from the current study.

Conclusions

Oral hyperplasia and dysplasia are associated with altered miR-21 and TAC expression. Salivary miR-21 are more accurate in detecting oral dysplasia than salivary TAC. Salivary miR-21 could be potential diagnostic biomarker for screening and early detection of oral cancer. More studies are required to validate the diagnostic power of miR-21.

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

Funding: Self-funded

References

1. Fedele S (2009) Diagnostic aids in the screening of oral cancer. Head Neck Oncol 1:5. doi: 10.1186/1758-3284-1-5
2. Naikmasur VG, Sattur AP, Mutalik S, Thakur AR (2009) Recent advances in diagnostic oral medicine. J Indian Acad Oral Med Radiol 21:99-104.
3. Macey R, Walsh T, Brocklehurst P, Kerr AR, Liu JL, Lingen MW, Ogden GR, Warnakulasuriya S, Scully C (2015) Diagnostic tests for oral cancer and potentially malignant disorders in patients presenting with clinically evident lesions. Cochrane Database Syst Rev 5:CD010276. doi: 10.1002/14651858.CD010276
4. Cervigne NK, Reis PP, Machado J, Sadikovic B, Bradley G, Galloni NN, Pintilie M, Jurisica I, Perez-Ordonez B, Gilbert R, Gullane P, Irish J, Kamel-Reid S (2009) Identification of a microRNA signature associated with progression of leukoplakia to oral carcinoma. Hum Mol Genet 18(24):4818-4829. doi: 10.1093/hmg/ddp446.
5. Chhabra N, Chhabra S, Sapra N (2015) Diagnostic modalities for squamous cell carcinoma: an extensive review of literature-considering toluidine blue as a useful adjunct. J Maxillofac Oral Surg 14(2):188-200. doi: 10.1007/s12663-014-0660-6.
6. Nagi R, Reddy-Kantharaj YB, Rakesh N, Janardhan-Reddy S, Sahu S (2016) Efficacy of light-based detection systems for early detection of oral cancer and oral potentially malignant disorders: Systematic review. Med Oral Patol Oral Cir Bucal 21(4):e447-455.
7. Lingen MW, Abt E, Agrawal N, Chaturvedi AK, Cohen E, D'Souza G, Gurenlian J, Kalmar JR, Kerr AR, Lambert PM, Patton LL, Sollecito TP, Truelove E, Tampi MP, Urquhart O, Banfield L, Carrasco-Labra A (2017) Evidence-based clinical practice guideline for the evaluation of potentially malignant disorders in the oral cavity: A report of the American Dental Association. J Am Dent Assoc 148(10):712-727.e10. doi: 10.1016/j.adaj.2017.07.032.
8. Radhika, Jeddy, Nithya, Muthumeenakshi (2016) Salivary biomarkers in oral squamous cell carcinoma – An insight. J Oral Biol Craniofac Res 6(Suppl 1):S51-S54.

9. Patil S, Arakeri G, Alamir AWH, Awan KH, Baeshen H, Ferrari M, Patil S, Fonseca FP, Brennan PA (2019) Role of salivary transcriptomics as potential biomarkers in oral cancer: A systematic review. J Oral Pathol Med 48(10):871-879. doi: 10.1111/jop.12895.
10. Korde SD, Basak A, Chaudhary M, Goyal M, Vagga A (2011) Enhanced nitrosative and oxidative stress with decreased total antioxidant capacity in patients with oral precancer and oral squamous cell carcinoma. Oncology 80(5-6):382-389. doi: 10.1159/000329811.
11. Vlková B, Stanko P, Minárik G, Tóthová L, Szemes T, Baňasová L, Novotňáková D, Hodosy J, Celec P (2012) Salivary markers of oxidative stress in patients with oral premalignant lesions. Arch Oral Biol 57(12):1651-1656. doi: 10.1016/j.archoralbio.2012.09.003.
12. Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E, Wong DT (2009) Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. Clin Cancer Res 15(17):5473-5477. doi: 10.1158/1078-0432.
13. Brito JA, Gomes CC, Guimarães AL, Campos K, Gomez RS (2014) Relationship between microRNA expression levels and histopathological features of dysplasia in oral leukoplakia. J Oral Pathol Med 43(3):211-216. doi: 10.1111/jop.12112.
14. Kaur J, Jacobs R, Huang Y, Salvo N, Politis C (2018) Salivary biomarkers for oral cancer and pre-cancer screening: a review. Clin Oral Investig 22(2):633-640. doi: 10.1007/s00784-018-2337-x.
15. Zahran F, Ghalwash D, Shaker O, Al-Johani K, Scully C (2015) Salivary microRNAs in oral cancer. Oral Dis 21(6):739-747. doi: 10.1111/odi.12340.
16. Hung KF, Liu CJ, Chiu PC, Lin JS, Chang KW, Shih WY, Kao SY, Tu HF (2016) MicroRNA-31 upregulation predicts increased risk of progression of oral potentially malignant disorder. Oral Oncol 53:42-47. doi: 10.1016/j.oraloncology.2015.11.017.
17. Agha-Hosseini F, Mirzaii-Dizgah I, Farmanbar N, Abdollahi M (2012) Oxidative stress status and DNA damage in saliva of human subjects with oral lichen planus and oral squamous cell carcinoma. J Oral Pathol Med 41(10):736-740. doi: 10.1111/j.1600-0714.2012.01172.x.
18. Babiuch K, Bednarczyk A, Gawlik K, Pawlica-Gosiewska D, Kęsek B, Darczuk D, Stępień P, Chomyszyn-Gajewska M, Kaczmarzyk T (2019) Evaluation of enzymatic and non-enzymatic antioxidant status and biomarkers of oxidative stress in saliva of patients with oral squamous cell carcinoma and oral leukoplakia: a pilot study. Acta Odontol Scand 77(6):408-418. doi: 10.1080/00016357.2019.1578409.
19. Shetty R, Kali A and Shetty R (2015) Serum total antioxidant capacity in oral carcinoma patients. Pharmacognosy Res 7(2):184–187. doi: 10.4103/0974-8490.150513
20. Pakfetrat A, Dalirsani Z, Hashemy S, Ghazi A, Mostaan LV, Anvari K, Pour AM (2018) Evaluation of serum levels of oxidized and reduced glutathione and total antioxidant capacity in patients with head and neck squamous cell carcinoma. J Cancer Res Ther 14(2):428-431. doi: 10.4103/0973-1482.189229.