DR. GAREEMA PRASAD (Orcid ID : 0000-0002-1316-7091)

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A PANEL OF MICRORNAS CAN BE USED TO DETERMINE ORAL SQUAMOUS CELL CARCINOMA

Gareema Prasad[1, 2], Christine Seers[1 2], Eric Reynolds[1, 2], and Michael J. McCullough [1, 2]*

[1] Melbourne Dental School, The University of Melbourne, Melbourne, VIC, 3053, Australia

[2] Oral Health Cooperative Research Centre, The University of Melbourne, Melbourne, VIC, 3053, Australia

* To whom correspondence should be addressed; Dr Gareema Prasad- Melbourne Dental School, The University of Melbourne, 720 Swanston Street, Carlton 3053 Victoria, Australia; Email: prasadgareema@hotmail.com; Phone- +61 3 93411490; Fax- +61 3 93411400

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Conflict of Interest

The authors of the manuscript titled: “A panel of microRNAs can be used to determine oral squamous cell carcinoma -,” certify that they have NO conflict of interest. They have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

Abstract

Background - Subjective histopathology is currently used to diagnose oral squamous cell carcinoma (OSCC). We tested if abundances of a panel of microRNA could be an objective OSCC indicator.

Method-Literature review enabled identification of 10 microRNAs associated with oral and head and neck malignancies. We extracted RNA from formalin-fixed paraffin embedded biopsies; 20 each with OSCC, dysplasia or histologically normal epithelium (HNE) and 10 with oral lichen planus (OLP). Relative abundances of microRNAs in HNE and OSCC were determined using reverse transcription then real time PCR with global mean normalization. MicroRNAs differentially expressed (test microRNA, T-miR) and non-differentially expressed (normalization microRNA, N-miR) were identified. The raw microRNA Cq data was incorporated in a developed algorithm that output a T-miR expression value (T-miREV) score. Raw Cq data from HNE, OSCC, dysplasia and OLP samples were then used to test the algorithm scoring and OSCC classification.

Results-Four test and normalisation microRNAs were identified. Algorithm output of T-mirEV >1 or <-1 indicated high and low OSCC probability score respectively and gave 88.9% sensitivity, 100% specificity and 93.5% accuracy. Grouping high and intermediate T-mirEV scores (T-miREV ≥-1) resulted in sensitivity of 90%, specificity of 65% and accuracy of 77.5% in OSCC classification. All 20 dysplasias and 8 of 10 OLP had T-miREV ≥-1 indicating intermediate to high probability of malignant changes.

Conclusion-A microRNA panel combined with our algorithm can identify tissue with probable oncogenic changes

Impact-The developed algorithm serves as a baseline for prospective trials which may result in potential clinical utility.

Introduction

The gold standard in diagnosis of malignant and potentially malignant oral mucosal lesions is incisional biopsy and histo-pathological assessment. However, histo-pathological examination has concerns related to sampling errors, subjective errors in interpretation, and a lack of sensitivity to determine if a lesion is likely to progress to malignancy [1, 2]. Hence, there is a need for a more objective system to predict the potential for a lesion to progress to cancer. There is significant work being undertaken to identify markers in patients with established oral cancer or disorders with the potential to transform to malignancy. However, up until now, no particular marker has been identified or algorithm constructed which has better prognostic value than histo-pathological analysis. Dysregulation of cancer associated microRNAs in OSCC induces cell proliferation and anti-apoptosis promotes cancer metastasis and potentiates resistance to chemotherapy [3]. MicroRNAs have been indicated to be potentially useful biomarkers in pathological tissue [[4-7]. The microRNA-regulated pathways in OSCC result in either upregulation of microRNAs or downregulation of microRNAs [5]. Over the past several years several studies have shown dysregulation of certain microRNA in oral cancer when compared with normal tissue [5,8-10]. Some studies have also shown that microRNAs are differentially expressed in differing severity of lesions and can potentially serve as biomarkers of dysplasia and OSCC [10]. Although dysregulated microRNAs have been identified [11-13], no single microRNA has been consistently identified in all studies. We propose that use of a combination of microRNAs that have been shown to be dysregulated in different studies may be of use due to a combinatorial effect.

In this study we aimed to assess the feasibility of utilizing microRNAs as a biomarker for differentiating between oral squamous cell carcinoma (OSCC), OLP, dysplasia and normal tissue.

Materials and methods

Ethics approval was obtained from the Human Research Ethics Committee, Melbourne Dental School, The University of Melbourne. Written informed consent from the patient (donor) was obtained for the use of this sample in research.

Selection of microRNA panel

Case controlled studies were identified from COCHRANE and PUBMED databases and microRNA selected for use in a microRNA panel were chosen from these. MicroRNA selected had to meet the criteria of (1) consistently dysregulated in most HNSCC studies, in particular OSCC; (2) dysregulated

in studies with large sample sizes (n>20); (3) dysregulated in studies where samples were tested by more than one method.

Sample collection

Formalin-fixed paraffin embedded (FFPE) biopsy samples were randomly selected from the Melbourne Dental School Oral Pathology Diagnostic Service archive. Samples comprised 20 OSCC, 20 histologically normal epithelium (HNE), 10 with mild dysplasia, 10 with moderate or severe dysplasia and 10 OLP. HNE were denture induced fibrous hyperplastic tissue. For the purpose of the present study, lesions with mild dysplasia were categorized separately to those lesions with moderate to severe dysplasia. This demarcation follows the WHO Collaborating Centre for Oral Cancer and Precancer recommendation [14].

RNA extraction and Real Time PCR

Four 20 µm thick sections were sliced from the FFPE blocks and RNA extracted using the Recover All™ Nucleic Acid Isolation Kit (Life Technologies). All extracted RNA was quantified by spectrophotometer, (NanoDrop® ND1000; NanoDrop Technologies, Wilmington, DE, USA) and quality examined using an Experion system (BioRad Laboratories). Reverse transcription (RT) of 50 ng of control human heart RNA(Life Technologies) or RNAs derived from FFPE tissues were performed in triplicate using the Megaplex™ Primers Human Pools A, with an additional preamplification step (Life Technologies). Real-time PCR using TaqMan hydrolysis probes (Life Technologies) was used to determine Cq. The Cq data were analyzed using qbase PLUS software (Version 2, Biogazelle 20082011), normalized using the global mean normalization (GMN) strategy and adjusted by the normalization value to give the calibrated normalized relative quantity (CNRQ).

Algorithm development

An unpaired Student's *t*-test was conducted on the CNRQ values. The microRNA determined to be dysregulated in OSCC ($p<0.05$ with no overlap of 95% the confidence intervals [CI]) were designated test microRNA (T-miR) and those not dysregulated were used as normalization microRNA (N-miR). The raw Cq values were used to generate the algorithm incorporating adjustment to compensate for sample to sample variation. We considered that if the Cq value for an N-miR in a sample was lower (or higher) than the N-miR mean Cq value for all samples combined, it indicated that total RNA sample had a skewed miR abundance and thus in that sample T-miR would also be overrepresented (or underrepresented). Therefore, the T-miR Cq values were adjusted for that sample in proportion to the N-miR (NAdj). The mean differences for all N-miR were used to derive a normalization value (NV) for each sample. Thus, the Cq value for each T-miR tested was

subsequently adjusted by the NV for that sample to give T-miR-norm Cq (Fig 1A). However, to compensate for the fact that each T-miR-norm had a different mean and standard deviation, the T-miR-norm was then converted into the number of standard deviations that sample T-miR-norm was from the mean T-miR-norm of the 20 HNE samples. This resulted in the T-miR-expression value (T-miREV) (Fig 1B).

Results

Selection of microRNA panel

From the selected literature (Appendix A), nine microRNAs reported to be dysregulated were selected to test, miR-21, miR-31, miR-127, miR-155, miR-197, miR-210, miR-24, miR-19b, miR-26b. In addition miR-205 was also selected as it had previously been assessed in a number of oral malignant lesions and found to be highly specific for squamous epithelium and not dysregulated and thus could potentially serve as a control [15].

MicroRNA comparative abundances in OSCC, HNE, dysplasia and OLP

TaqMan hydrolysis probes were used in qPCR to determine abundances of microRNAs in OSCC and HNE RNA. The normalization factors calculated varied greatly ranging from 0.014 AU (arbitrary units) for samples No. 173 and 120 and up to 39.24 AU for sample No. 120 with an even distribution of AU values above or below 1 (Appendix B).

Comparison of the CNRQ of microRNA of OSCC with HNE (unpaired Student's *t*-test: $p<0.05$ and 95% CI not overlapping) showed that miR-24, miR-26b and miR-155 were down-regulated and miR-21 up-regulated. These microRNA were selected as T-miRs. Four microRNA met the $p>0.05$ significance criterion (unchanged), miR-19b, miR-31, miR-205 and miR-210 and were used as N-miRs. Notably in dysplasia and OLP miR-24, miR-26b and miR-155 were also down-regulated, suggesting these microRNA are indicators of change in the epithelium.

Defining algorithm metrics

To define OSCC a T-miREV value >1.0 was taken as an indication of high probability of OSCC, whilst a T-miREV value of <-1.0 was taken as an indication for low probability of OSCC. T-miREV

values ≥-1.0 to ≤1.0 were considered in the intermediate category (Table 2). In a strictly positive-negative analysis samples of intermediate score were ignored in the output.

Of the 20 OSCC samples 16 were calculated to have a positive OSCC score and 2 to have a negative OSCC score (Table 2). No HNE sample had a positive OSCC score and 13 were categorized as negative for OSCC (Table 2). This results in sensitivity of 88.9%, specificity of 100%, and an accuracy of 93.5%.

However, to have clinical relevance the intermediate category needs to be considered as there can be a spectrum of changes indicative of epithelial dysplasia that may represent an early indication of oral cancer. When sensitivity and specificity is calculated grouping samples with high and intermediate score (T-miREV ≥-1) versus low OSCC score (T-miREV <-1) the result is sensitivity of 90%, specificity of 65% and an accuracy of 77.5% (Table 2). The reduction in sensitivity, specificity and accuracy derived from capture of HNE samples in the intermediate risk category.

Utilizing the miR-OSCC-risk algorithm to examine the dysplasia samples revealed that all lesions with dysplasia had an intermediate to high miR-OSCC-risk. When the results of OSCC and dysplasia are pooled, 36 out of the 40 lesions are in the intermediate to high miR-OSCC-risk category (Table 3). Furthermore, the majority of OLP samples (8 of 10) were also in the intermediate to high miR-OSCC-risk category (Table 3). Thus the use of the T-miR and N-miR combination determined by this study in combination with the developed algorithm enables detection of oral abnormalities.

Discussion

The present study undertook a systematic literature search to select microRNA to test for abundance variations between HNE and OSCC. We selected microRNA from studies which tested and confirmed the dysregulation of microRNAs by more than one method (e.g. microarray and qPCR) and eliminated bias by selecting microRNAs that were consistently dysregulated in 2 or more HNSCC studies, in particular OSCC. The microRNAs shortlisted for the panel were from several tissue types-OSCC cell lines, frozen tissues, patient saliva and FFPE (Appendix A). Additionally the cancers were from various oral sites, HNSCC, tongue SSC and nasopharyngeal. Using this method we identified 10 microRNAs that may be of relevance to OSCC. Eight were subsequently found to be useful in a microRNA panel, four as dysregulated biomarkers and four as non-dysregulated control biomarkers.

We found wide variation in the content of microRNA present in the RNA extracts from FFPE biopsies (Appendix B). This is a factor difficult to avoid due to inherent sample variance such as lesion sizes and surgical sampling. Thus normalization is especially important for valid sample to

sample comparisons. This is a consistent problem faced by all methodologies that use biomarker variances rather than positive/negative indicators. The strategy used here was designed to compensate for these variances. GMN was considered most apt for data normalization as it does not rely on one chosen control remaining invariant in all test conditions.

OLP is a chronic mucocutaneous inflammatory disease more often affecting older females (Pendyala, Joshi *et al.* 2012). The sample is representative of this cohort as 9 of the 10 OLP patients in the present study were female between the ages of 50 – 75 years. Notably OLP had 2 of 10 samples in the high OSCC score category and 6 of intermediate score. This high risk scoring of OLP is similar to that of severe oral epithelia dysplasia. It can be postulated that the inflammatory nature of OLP is the cause of dysregulation of these microRNAs. A study by Danielsson *et al* (2012) reports decreased expression of miR-26b associated with increased expression of cyclooxygenase-2, a protein connected to inflammation that is involved in OLP. The results of this present study show that miR-26b was down regulated in OLP when compared to HNE samples by a ratio of 7.48 (Table 1).

The microRNAs dysregulated in dysplasia and OSCC were similar with the exception of miR-127 which was found to be upregulated in dysplasia and OLP. It is possible that miR-127 is upregulated during lesion progression or alteration in squamous cell differentiation, but its expression decreases once the carcinoma is established. Thus this microRNA may serve as a marker of progressive lesions from HNE to OLP or dysplasia then to OSCC.

On average in OSCC and dysplasia miR-21 was down-regulated relative to HNE. Discrimination between OSCC, dysplasia and OLP was indicated by the pattern of up-regulation of miR-21, miR-127 and miR-197. In dysplasia both miR-21 and miR-127 were increased relative to HNE (3.17-fold and 14.87-fold respectively) whereas in OSCC only miR-21 had increased abundance relative to HNE (6.25-fold) suggesting miR-127 may be "switched off" when OSCC is established. In OLP miR-21 was not increased in abundance relative to HNE, rather miR-127 (13.17-fold) and miR-197 (4.40-fold) were upregulated (Table 1). MiR-21 was twice as upregulated in OSCC than in dysplasia, miR-24 was almost three times more down regulated in OSCC than in dysplasia and miR-155 was almost three times more highly down regulated in dysplasia than OSCC. This differential expression of miR-155, miR-24 and miR-21 in OSCC relative to dysplasia and the lack of significance (or switching off) of miR-127 in OSCC compared to dysplasia may be hallmarks of progression of lesions from dysplasia to OSCC. The lack of dysregulation of miR-21 in OLP with the up-regulation of miR-197 may be a marker of OLP as a benign rather than progressing lesion. Larger multi-center analyses with longitudinal data are required to draw definitive conclusions.

For clinical relevance, the indeterminate T-miREV ($\geq$-1, $\leq$1) need to be taken into account as all suspect lesions should be flagged for further investigation. In such situations the high sensitivity is

of critical importance (90% in this study; Table 2) and the accuracy in detecting normal tissue is not of as critical clinical relevance. Despite the lower specificity of the algorithm including T-miREV ≥-1 (65%) the algorithm gave high sensitivity and accurately detected cancerous tissue. Thus, using this clinically relevant assessment few OSCCs would escape the requirement of a biopsy. It is critical to further analyze this panel of microRNAs prospectively with clinical samples and compare the results from the panel and algorithm to the histological assessment.

In the present study statistical analysis was conducted combining mild, moderate and severe dysplasia in comparison with OSCCs followed by only moderate to severe dysplasia with OSCCs. Interestingly, both combinations yielded the same specificity (65%). When mild dysplasia was omitted from the analysis, the sensitivity dropped from 95% to 93.3%, and the accuracy decreased from 85% to 82% (Appendix C). Thus there is very little difference in the miR-OSCC-risk values when mild dysplasia is included or omitted. This is consistent with the qbase analysis (Table 1) which showed no statistical significance (p >0.05) between mild and moderate to severe dysplasia. This is supported by a study conducted by Holmstrup *et al* (2006), which assessed the long-term outcome of potentially malignant lesions [1]. Holmstrup *et al* reported that surgically treated lesions with mild, moderate, severe dysplasia or no dysplasia developed carcinomas with similar frequencies of 9-11% [1], concluding that the degree of epithelial dysplasia was not a statistically significant factor for malignant development [1].

It is of interest to note that all samples in the dysplasia category were considered as high risk. Additionally, there were 7 HNE samples that were considered to be of intermediate risk. The tissues tested here were subject to biopsy because there was a clinical feature detected that flagged them for examination. Samples in the dysplasia category examined herein scored as possibly malignant and 7 HNE samples were considered to be in the intermediate category. It may be that the methodology employed here has enabled the detection of lesions prior to the presentation of the gross morphological changes needed for classification by visual examination by a pathologist. It may also be that the HNE cases classified as intermediate risk demonstrate that the algorithm has limitations in ruling out HNE samples as it is not just sensitive to dysplasia but may also be sensitive to inflammatory changes. Temporal expression of microRNA may also explain the seeming discrepancy between risk classification and diagnosis. Initiation of lesion development can correlate with changes in microRNA expression which can revert once malignant transformation has eventuated. Long term follow up of these patients in order to fully assess malignant development would be valuable to this study.

Using the developed algorithm to indicate possible malignancy with the high, intermediate or low criteria could be a simple way for clinicians to accurately and objectively assess mucosal tissue as an adjunct to histopathology. The algorithm developed here is not a prognostic marker test but instead is

used to determine if OSCC, dysplasia, OLP and HNE can be differentiated. To be clinically relevant as a diagnostic test, this study needs to be conducted prospectively with comparison to the gold standard histology.

At present the algorithm has been only used with miR abundances indicated in tissues obtained by biopsy. Development of the method as a "chair-side tool" would be facilitated by streamlining of the method of sample acquisition (e.g. alternatives to full biopsy), sample processing and subsequent PCR to produce results in a timely manner.

Conclusion

A microRNA panel and algorithm was developed that enabled the discrimination of oral lesions with OSCC from normal tissue. This lays the groundwork for future prospective studies to assess oral cancer risk and differentiate OSCC from other lesions and potentially serve as an objective adjunct to oral histopathological diagnosis of lesions. This microRNA panel and algorithm may in the future have clinical utility as a prospective chair-side tool including the assessment of unknown clinical lesions, margins of OSCC for excision, the recurrence of oral cancer and for the assessment of other potentially malignant lesions.

DISCLAIMER

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Table 1: The fold changes of the mean microRNA CNRQ significantly different between tissues with different histopathological diagnoses.

Tissue comparison[a]	Ratio of CNRQ					
	miR-155	miR-24	miR-26b	miR-21	miR-127	miR-197
OSCC/HNE				**6.25**		
HNE/OSCC	**8.67**	**10.63**	**2.58**			
MD/SD						
SD/MD						
*dysplasia/HNE				**3.17**	**14.87**	
HNE/dysplasia	**26.65**	**3.74**	**7.23**			
OLP/HNE					**13.17**	**4.40**
HNE/OLP	**7.10**	**3.45**	**7.48**			
dysplasia/OLP						
OLP/dysplasia	**3.76**					
dysplasia/OSCC					**8.95**	
OSCC/dysplasia			**2.77**			

a. OSCC, oral squamous cell carcinoma (n=20); HNE, histologically normal epithelium (n=20); MD, mild dysplasia (n=10); SD, moderate and severe dysplasia (n=10); dysplasia, all levels of dysplasia (n=20); OLP, oral lichen planus (n=10). Only the fold changes that were statistically significantly different (Unpaired t-test: $p<0.05$ and 95% CI not overlapping) are shown.

** No significant differences were identified between the CNRQs of lesions with mild dysplasia or moderate to severe dysplasia. Therefore the CNRQ data for lesions diagnosed with mild dysplaia or moderate-severe dysplasia were pooled and referred to and analysed collectively as dysplasia*

Table 2: Assessment of OSCC status for OSCC vs HNE tissue

	Sample Type	miR-OSCC-risk		Sensitivity (%)	Specificity (%)	Accuracy (%)
		Positive	Negative			
High vs Low probability (T-mirEV >1 vs T-mirEV<-1)	OSCC	16	2	88.9	100	93.5
	HNE	0	13			
High and Intermediate vs Low probability (T-mirEV ≥-1 vs T-mirEV<-1)	OSCC	18	2	90.0	65.0	77.5

Table 3: OSCC classification of tissue with various pathologic diagnoses.

T-miREV	HNE[1]	OSCC[2]	MD[3]	SD[4]	OLP[5]
>+1 high probability	0	16	9	2	2
between +1 and -1 medium probability	7	2	1	8	6
<-1 low probability	13	2	0	0	2
Total	**20**	**20**	**10**	**10**	**10**

[1]*HNE- Histologically normal epithelium*

[2]*OSCC-Oral squamous cell carcinoma*

[3]*MD-Mild dysplasia*

[4]*SD-Moderate to severe dysplasia*

[5]*OLP-Oral mucosal lichen planus*

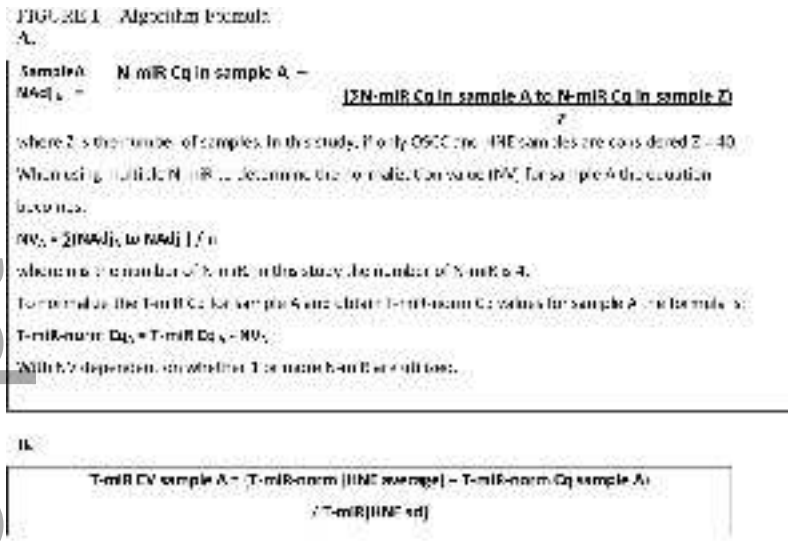

Fig 1. Normalization of Cq and description of T-MiR Expression Value. *A. The Cq value for each N-miR in each sample were adjusted to take into consideration the level of abundance of miR in that sample. This was undertaken by considering for each N-miR the number of cycles that was above the miR Cq differed for that sample from the overall mean Cq for the 40 control samples. When more than one N-miR is used (in this study we used four N-miR), the average difference that each of these N-miR varied from the sample N-miR is used to calculate the variance of abnormality and then used as a normalization value (NV) for that sample. B. To compensate for the fact that each T-miR-norm have different mean and standard deviations, the number of standard deviations a sample T-miR-norm was from the mean T-miR-norm of the 20 HNE (control) samples was calculated. This measure, the T-miR-expression value (T-miR- EV). The T-miR- EV is the number of standard deviations a sample T-miR-norm Cq is from the mean Cq of the HNE samples T-miR norm (T-miR norm/(HNE average)).*

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