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Non-invasive screening of a microRNA-based dysregulation signature in oral cancer and oral potentially malignant disorders

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ABSTRACT

Introduction: We have previously shown that oral swirls are a robust source of microRNA protected by extracellular vesicles, potentially useful to detect oral squamous cell carcinoma (OSCC)-associated molecular aberration.

Objectives: To study a developed dysregulation score and risk classification algorithm based upon a panel of OSCC-associated microRNA in oral swirls from individuals with OSCC and oral potentially malignant disorders (OPMDs).

Materials and methods: An OSCC-associated panel of 5 microRNAs (miR-24; miR-21; miR-99a; let-7c; miR-100;) was quantified by qPCR in 190 individuals with and without mucosal abnormalities, including OSCC (n = 53) and OPMDs (n = 74). Each sample was analyzed using a developed dysregulation score (dSCORE) and risk classification algorithm, allocating a LOW- or HIGH-RISK score. The influence of demographic, systemic, oral health and mucosal disease factors on the developed test was analyzed.

Results: MicroRNA for analysis can be predictably isolated from oral swirls sourced from individuals with a range of demographic, systemic and oral health findings. Utilizing the presence of HIGH-RISK identified OSCC patients with 86.8% sensitivity and 81.5% specificity. Older age and female gender were associated with higher dSCOREs and higher proportions of HIGH-RISK classification amongst individuals with no mucosal abnormalities. The dSCOREs for all subgroups of OPMDs were significantly different from the OSCC group.

Conclusion: This is the first comparison of microRNA sourced from oral swirls from individuals with OPMDs with individuals with and without OSCC. A HIGH-RISK dysregulation signature was found to be accurate in indicating the presence of OSCC and exampled to parallel malignant transformation.

Introduction

Late stage oral squamous cell carcinoma (OSCC) is associated with significant rates of morbidity and mortality. In contrast, early stage OSCC has a favourable prognosis with a five-year survival rate of over 80% [1]. Although screening for pre-cursor lesions or oral potentially malignant disorders (OPMDs) increases the chance of early stage diagnosis [1], there is currently limited capacity for prediction of an individual's development of malignancy. The most common OPMDs are leukoplakia and oral lichen planus (OLP) or oral lichenoid lesions (OLL). The global prevalence of all OPMDs is approximately 4.5% [2] and the overall malignant transformation rate is approximately 3–4.3% [3,4]. The combination of conventional oral examination and the presence of histopathological dysplasia remains the best indicator of potential for malignant progression [5].

Cells within the body secrete extracellular vesicles, reflecting their pathophysiological state, to communicate with neighbouring and distant cells [6]. OSCC cell-derived extracellular vesicles have been shown to confer pro-metastatic behaviour in neighbouring cells by delivery of microRNA [7]. Extracellular vesicles are in biofluids including saliva or

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gingivocrevicular fluid and have been highlighted as early and progressive biomarkers of disease course [8]. We have previously shown that oral swirls are a robust source of microRNA protected by extracellular vesicles [9]. The vesicle protection means that there is diminished need to have samples promptly frozen for microRNA study and do not require any special transport medium.

We identified a panel of OSCC-associated microRNAs, miR-24-3p, miR-21-5p, let-7c-5p, miR-99a-5p and miR-100-5p for study in oral swirls. Using a developed test combining a dysregulation score (dSCORE) and predictive algorithm, we identified the presence of OSCC with high accuracy using the abundance of these microRNA isolated from oral swirls [10].

This study explores the utility of the developed test in a cohort of individuals with and without mucosal abnormalities to determine the algorithm- based classification of OSCC risk for OPMDs. Furthermore, the influence of demographic, local and systemic factors on the developed test was examined.

Materials and Methods

The patient cohort

This study was approved by the Human Research Ethics Committee of the University of Melbourne (Ethics ID: 050900X) and Melbourne Health (Ethics ID: 2014.111). Patients (total n = 190) were from the Royal Dental Hospital of Melbourne or Royal Melbourne Hospital. Individuals included those with visible oral mucosal abnormalities (MA) who were attending for their oral mucosal condition (n = 136) and those with no mucosal abnormalities (NMA) who were attending for dental needs (n = 54). Patients with MA included those with histologically normal epithelium (HNE), such as a fibroepithelial polyp or denture associated hyperplasia, oral potentially malignant disorders (OPMD) or OSCC (Table 1).

The OPMD group included those with:

a. Oral lichen planus (OLP): clinically and histopathologically consistent with oral lichen planus with no evidence of dysplasia.
b. Oral lichenoid lesion (OLL): only either clinically or histopathologically suggestive of oral lichen planus but not both.
c. Oral leukoplakia without dysplasia: white lesion of questionable risk having excluded other known diseases or disorders with no evidence of dysplasia.
d. Dysplasia: any grade of epithelial dysplasia (mild, moderate, severe).
e. Traumatic ulceration with stromal eosinophils (TUGSE) with no evidence of dysplasia.

Patient data was recorded including presence of any comorbidities, smoking status (current, past or never), consumption of alcohol (daily, non-daily), dentate vs edentulous status and the presence of a removable prosthesis. For control patients (NMA) status of caries requiring intervention and the presence of gingivitis or periodontitis was recorded.

Oral swirl sample collection

The patients were instructed to swirl 10 mL of sterile deionized water for 50–60 s and expectorate into a sterile container. For patients with mucosal abnormalities, the oral swirl collection took place immediately before their diagnostic biopsy or between diagnostic biopsy and surgical resection.

Detection of Candida

Oral swirls from 120 patients (80 MA and 40 NMA) were tested for presence of *Candida* species by culture. The swirls were vortexed for 30 s for homogeneity and 100 µL plated on Sabouraud dextrose Agar (SDA) containing chloramphenicol and incubated at 37 °C for 48 h. If colonies were visualized, the patients were indicated as positive for *Candida* presence.

Table 1
Characteristics of total cohort (n = 190) by diagnostic category.

	Diagnostic Category			
	OPMD (n = 74)	OSCC (n = 53)	HNE (n = 9)	NMA (n = 54)
Characteristic				
Mean Age yrs ± s.d.	61.6 ± 14.1	61.4 ± 16.6	60 ± 7.5	46.5 ± 21.3
		n(%)		
Gender				
Male	42 (56.8)	29 (54.6)	1 (11.1)	26 (48.1)
Female	32 (43.2)	24 (45.3)	8 (88.9)	28 (51.9)
Tobacco Smoking				
Current	22 (29.7)	20 (37.7)	3 (33.3)	15 (27.7)
Past	36 (48.7)	23 (43.4)	4 (44.4)	33 (61.1)
Never	16 (21.6)	10 (18.9)	2 (22.2)	6 (11.1)
Daily Alcohol Consumption				
Yes	19 (25.7)	22 (41.5)	1 (11.1)	5 (9.2)
No	55 (74.3)	30 (56.6)	8 (88.9)	49 (90.7)
Not recorded	0	1 (1.9)	0 (0.0)	0 (0.0)
Comorbidities				
Yes	56 (75.7)	40 (75.5)	6 (66.7)	20 (37.0)
No	18 (24.3)	13 (24.5)	3 (33.3)	34 (63.0)
Dentate				
Yes	67 (90.5)	37 (69.8)	8 (88.9)	52 (96.3)
No	7 (9.5)	9 (17.0)	1 (11.1)	2 (3.7)
Not recorded	0 (0.0)	7 (13.2)	0 (0.0)	0 (0.0)
Removable Prosthesis				
Yes	24 (32.4)	12 (22.6)	3 (33.3)	11 (20.4)
No	50 (67.6)	34 (64.2)	9 (66.7)	43 (79.6)
Not recorded	0 (0.0)	7 (13.2)	0 (0.0)	0 (0.0)

OPMD – oral potentially malignant disorder; OSCC – oral squamous cell carcinoma; HNE – histologically normal epithelium; NMA – no mucosal abnormalities; s.d. - standard deviation.

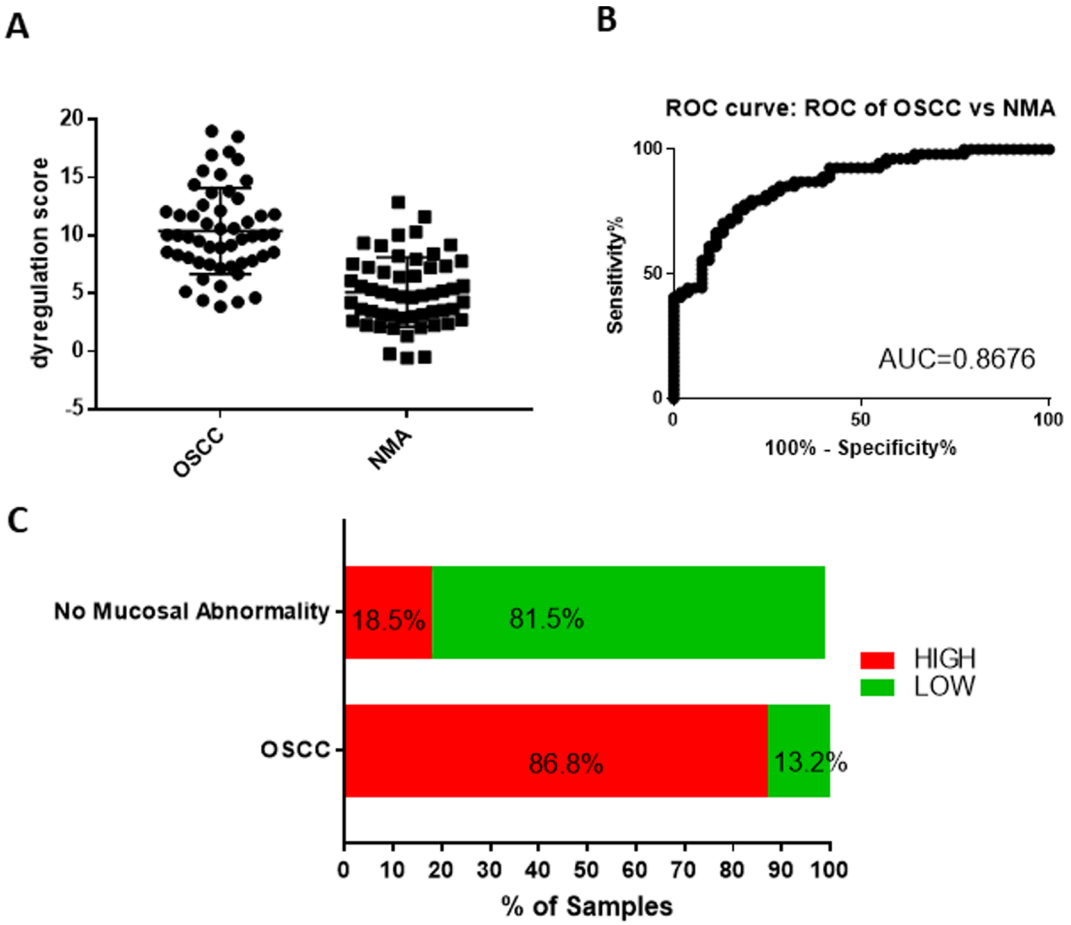

Fig. 1. Relationship of OS microRNA dSCOREs and RISK classification with the presence of OSCC. (A) dSCOREs for OSCC (n = 53) and NMA groups (n = 54) ($p < 0.001$); (B) Specificity and sensitivity ROC curve of dSCOREs for OSCC malignant and NMA groups; (C) Algorithm RISK assessment of NMA and OSCC demonstrating 86.8% sensitivity and 81.5% specificity.

Processing of oral swirls

The oral swirls were processed as previously described. Briefly, oral swirls were centrifuged at 4000*g* for 4 min at 4 °C to pellet epithelial cells, bacteria and large debris, which were discarded and the resultant cell-free swirl (OS) was stored in 1 mL aliquots at −20 °C. Thawed OS (5 × 1 mL) were combined with a prepared PEG-based solution following a previously described protocol [10] to enrich for EVs and the total RNA extracted using the mirVana™ microRNA isolation kit (Life Technologies) and measured by spectrophotometer (NanoDrop1000; NanoDrop Technologies, Wilmington, DE, USA). The RNA was concentrated using a Speedyvac™ concentrator or diluted with pyrocarbonatediethyel (DEPC) water to allow a maximum of 80 ng of total RNA into the reverse transcription reaction, according to the manufacturer's instructions.

Quantification by RT-qPCR

The microRNAs (miR-24-3p, miR-21-5p, let-7c-5p, miR-99a-5p, miR-100-5p) were quantified using reverse transcription quantitative PCR (RT-qPCR) with a custom multiplex primer pool and the EPIK™ microRNA Select Assay kit (Bioline, Eveleigh, NSW, Australia). The primers were as follows:

hsa-miR-99a-5p————AAC-CCGUAGAUCCGAUCUUGUG——
hsa- miR-100-5p ————AAC-CCGUAGAUCCGAACUUGUG———
hsa-let-7c-5p ——UGAGG-UAG————UAGGUUGUAUGGUU
hsa-miR-24-3p UGGCUCAGUUCAGCAGG-AACAG————————
hsa-miR-21-5p ———UAGCUUAUCAGA-CUGAUGUUGA————

The qPCR was performed in duplicate on an Eco, Illumina PCR machine. Human heart RNA (Life Technologies, Mulgrave, VIC, Australia) was used as a positive RNA control.

Dysregulation Score (dSCORE)

A dysregulation score (dSCORE, as previously described [10] was calculated for each sample analyzed. This was the cumulative variation of the qPCR C_t of each of test microRNAs from the C_t of the reference microRNA miR-99a-5p.

Algorithm-assessed risk (RISK)

The previously developed algorithmic decision tree used parameters as below to categorize each sample into LOW or HIGH-RISK of the presence of OSCC [10].

IF let-7-5p (< −1.00, "HIGH") (> 3.38, "LOW") otherwise ("RISK")

IF miR-21-5p (> 3.22, "HIGH") (< 0.745, "LOW") otherwise ("RISK")

IF miR-100-5p (< -6.98, "HIGH") (> 2.90, "LOW") otherwise ("RISK")

IF dSCORE (< 7.15, "LOW") otherwise "HIGH"

Statistical plan and analysis

Data were entered through *M Form 2.9*.1. Sensitivity analysis was undertaken to determine algorithm validity compared to the gold standard (clinical examination for the NMA group and histopathology for the MA group). Statistical analyses were performed and visualized using R 3.3.3 [11] and GraphPad Prism 7.03. Correlation between patient characteristics and outcomes using the dSCORE and -RISK algorithm was assessed using chi-square test for categorical data and t-tests or ANOVA or regression analyses for continuous data.

Results

Consistent RNA yield from OS

The mean total RNA yield of all 190 OS was 7.0 ± 8.3 ng/μL. Total RNA yield was not significantly affected by gender, age, presence of a comorbidity, tobacco smoking status or daily alcohol consumption. There was no difference found in RNA yield between individuals who were dentate or edentulous, those who had active caries, caries experience, caries risk, gingivitis or periodontitis or positive *Candida* culture (Supplementary Tables 1 & 2). The use of a removable prosthesis was associated with significantly lower RNA yield (ANOVA $p = 0.036$). No difference in RNA yield was found when comparing patients with and without mucosal abnormalities, no differences in RNA yield was observed amongst patients with different categories of mucosal abnormalities (Supplementary Table 1).

The relationship between dSCORE and -RISK and presence of OSCC

The dSCORE distribution showed a significant difference between patients with OSCC and NMA ($p < 0.001$) **(**Fig. 1**A)**. A ROC curve to assess sensitivity and specificity of the dSCORE had an AUC of 0.868 **(**Fig. 1**B)**. All samples were placed though the RISK assessment decision-tree algorithm categorizing each as either LOW- or HIGH-RISK. It was found that a HIGH-RISK signature was present in 46/53 (86.8%) of the OSCC group and 10/54 (18.5%) of NMA **(**Fig. 1**C)**. This resulted in a sensitivity of 86.8% (95% C.I. 84.5–88.1), specificity of 81.5% (95% C.I. 79.0–83.9) and accuracy of 84.2 (95% C.I. 82.5–85.7).

The OSCC group included individuals with both small and large tumours, early and advanced disease. No evidence of difference in dSCORE or number of patients with HIGH-RISK was observed in relation to tumour size, site, presence of nodes, stage or differentiation **(**Table 2**)**.

The mean dSCORE of the HNE and OPMD groups were not different from the NMA group (ANOVA $p = 0.722$). However, the mean dSCORE for the OSCC group was significantly higher than all other groups (ANOVA $p < 0.001$) **(**Fig. 2**)**. The group of mucosal disorders that were not OSCC: HNE, leukoplakia, dysplasia, OLP, OLL and TUGSE showed no statistically significant differences in their mean dSCORE (ANOVA $p = 0.504$). The dSCORE for all subgroups of OPMDs were significantly different from the OSCC group (ANOVA post-hoc Tukey's *t*-test $p < 0.001$), except TUGSE, however this subgroup had only a limited number ($n = 2$).

All categories of mucosal abnormalities had a proportion of both HIGH-RISK and LOW-RISK individuals within each group. Of the OPMDS, the group with the lowest HIGH-RISK prevalence was the 'leukoplakia with no dysplasia' group, with a prevalence of 23.1% and the group with the highest HIGH-RISK prevalence was the 'dysplasia' group, with a prevalence of 46.2% **(**Table 3**)**.

The effect of age and gender on dSCORE and -RISK

Comparing dSCORE to age, logistic regression showed an interaction between increasing age and increasing dSCORE in the NMA group ($p = 0.001$) **(**Fig.3**A)**, which was not seen in the OSCC group **(**Fig. 3**B)**. An ANOVA and post-hoc Tukey's *t*-test found that NMA individuals below 50 years old had significantly lower mean dSCORE when compared to NMA individuals above 50 years (*t*-test $p = 0.016$). Both NMA age group dSCOREs were found to be significantly different from both OSCC age groups (ANOVA $p = 0.031$). Using HIGH-RISK to predict the presence of OSCC, it was found that age did not affect the sensitivity of the test (chi-square $p = 0.746$), however, specificity increased in older age groups (chi-square $p < 0.001$).

Comparing the dSCORE of males and females, ANOVA and post-hoc Tukey's test found no significant difference between males and females within the NMA ($p = 0.289$) or the OSCC groups ($p = 0.719$).

The ability of the RISK algorithm to identify OSCC was not significantly different between the genders (chi-square $p = 0.060$), however, the specificity was significantly higher in the male group (92.3%) than the female group (71.4%) (chi-square $p = 0.048$).

A linear model within the NMA group showed that there were independent effects of both older age ($p < 0.001$) and gender ($p = 0.008$), with older females associating with higher dSCOREs **(**Fig. 3**C)**. Similar observations of female gender and older age were not found within the OSCC group **(**Fig. 3**D)**. When combining factors of both female gender and age 50 + years, 7/11 (63.6%) of the NMA had a HIGH-RISK classification, thus a high false positive rate in this particular group. In contrast, 16/17 (94.1%) NMA females aged below 50 years, were correctly identified as LOW-RISK.

Within the OPMD group, there was no difference in mean dysregulation score between < 50 years and 50 + years (chi-square $p = 0.8841$) and no difference in prevalence of HIGH-RISK signature in < 50 years and 50 + years age subgroups of the HNE (*chi-square* $p = 0.264$) and OPMD groups (chi-square $p = 0.521$).

The mean dSCORE was not different for gender subgroups within the OPMD (*t*-test $p = 0.455$) groups. There was no difference in the prevalence of HIGH-risk signature in gender subgroups of the HNE (chi-square $p = 0.632$) and OPMD groups (chi-square $p = 0.257$).

Comorbidity, risk-related habits and dSCORE and -RISK

Within the NMA group, the presence of a comorbidity did not affect the dSCORE independent of age (linear model $p = 0.240$) or the sensitivity (chi-square $p = 0.226$) or specificity (chi-square $p = 0.0957$) of RISK.

There was no difference in mean dysregulation score vs presence of any comorbidity for the HNE group (*t*-test $p = 0.190$) or OPMD group (*t*-test $p = 0.829$). There was no difference in the prevalence of HIGH and LOW-RISK between individuals with and without a comorbidity in the HNE (chi-square $p = 1.00$) or OPMD group (chi-square $p = 0.378$).

There was no independent effect of smoking status (ANOVA post-hoc Tukey's *t*-test $p = 0.706$) or daily alcohol consumptions (ANOVA post-hoc Tukey's *t*-test $p = 0.502$) on the difference in dSCORE between the NMA and OSCC group or on the sensitivity or specificity of the algorithm-assessed risk (chi-square $p = 0.298$).

There was no difference in mean dysregulation score vs smoking status or daily alcohol consumption for the HNE group (Smoking status ANOVA $p = 0.248$; Daily Alcohol n/a) or OPMD group (Smoking Status ANOVA $p = 0.709$; Daily Alcohol *t*-test $p = 0.617$). There was no difference in prevalence of HIGH and LOW RISK between current, never and past smokers in the HNE (chi-square smoking status $p = 0.659$; daily alcohol $p = 0.632$) group or OPMD group (chi-square smoking status $p = 0.8591$; daily alcohol $p = 0.563$).

Patient dentition, periodontal status, Candida carriage and dSCORE and -RISK

Linear modelling found no difference in dSCORE between edentulous and dentate individuals, controlled for age, in the NMA ($p = 0.238$), OSCC ($p = 0.168$) and OPMD group ($p = 0.741$). In individuals with no mucosal abnormalities, there was no difference in

Table 2
TNM and Stage Subsets of OSCC (n = 53).

TNM and Stage	n (% of 53)	dSCORE mean ± s.d.	ANOVA	n of row total (%) HIGH	LOW	Chi-square
Tumour						
T1	24 (45.3)	10.6 ± 3.72	p = 0.461	20 (83.3)	4 (16.7)	p = 0.863
T2	10 (18.9)	8.76 ± 3.16		8 (80.0)	2 (20.0)	
T3	3 (5.7)	9.71 ± 2.23		3 (100.0)	0 (0.0)	
T4a	15 (28.3)	11.11 ± 4.29		14 (93.3)	1 (6.7)	
not specified	1 (1.9)	11.00		1 (100.0)	0 (0.0)	
Nodes						
Yes	14 (71.7)	10.75 ± 3.65	p = 0.884	12 (85.7)	2 (14.3)	p = 0.920
No	38 (26.4)	10.20 ± 3.81		33 (86.8)	5 (13.2)	
not specified	1 (1.9)	11.00		1 (100.0)	0 (0.0)	
Stage						
1	22 (41.5)	10.50 ± 3.83	p = 0.738	18 (81.8)	4 (18.2)	p = 0.814
2	6 (11.3)	8.34 ± 2.89		5 (83.3)	1 (16.7)	
3	5 (9.4)	10.89 ± 2.37		5 (100.0)	0 (0.0)	
4	19 (35.8)	10.66 ± 4.16		17 (89.5)	2 (10.5)	
not specified	1 (1.9)	11.00		1 (100.0)	0 (0.0)	
Differentiation						
poorly	5 (9.4)	8.55 ± 4.77	p = 0.587	3 (60.0)	2 (40.0)	p = 0.438
moderately	22 (41.5)	10.49 ± 3.69		20 (90.9)	2 (9.1)	
well	16 (30.1)	10.99 ± 4.22		14 (87.5)	2 (12.5)	
verrucous	2 (3.8)	12.46 ± 2.70		2 (100.0)	0 (0)	
not specified	8 (15.1)	9.35 ± 1.73		7 (87.5)	1 (12.5)	
Site						
lateral tongue	17 (32.1)	9.28 ± 3.84	p = 0.333	13 (76.5)	4 (23.5)	p = 0.345
floor of Mouth	15 (28.3)	11.44 ± 3.49		14 (93.3)	1 (6.7)	
alveolus	13 (24.5)	9.79 ± 2.76		19 (90.5)	2 (9.5)	
buccal mucosa	5 (9.4)	11.32 ± 5.24				
retromolar trigone	2 (3.8)	9.64 ± 4.22				
palate	1 (1.9)	17.20				

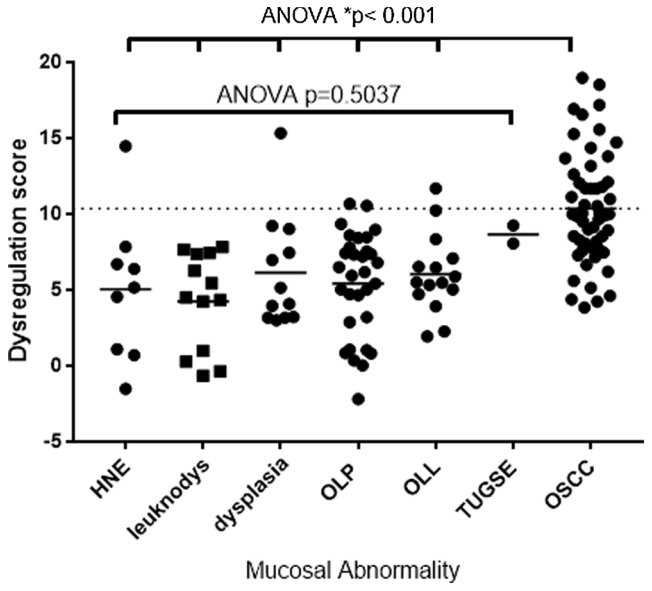

Fig. 2. Dysregulation score of non-OSCC mucosal abnormalities were not significantly different (p = 0.504). Dysregulation score of HNE and OPMDs (except TUGSE (n = 2)) were significantly different from OSCC (*p < 0.001).

dSCORE found in comparing individuals with or without a removable prosthesis (*t*-test p = 0.123), caries requiring intervention (ANOVA p = 0.811), past caries (ANOVA p = 0.865), caries risk (ANOVA p = 0.752), gingivitis (ANOVA p = 0.357) or periodontitis requiring intervention (ANOVA p = 0.647). Chi-square analysis found no evidence of difference in HIGH-RISK prevalence between subgroups of NMA for caries requiring intervention (p = 0.953), caries experience (p = 0.688), caries risk (p = 0.945), gingivitis (p = 0.348), or periodontitis requiring intervention (p = 0.735).

Table 3
HIGH- and LOW- RISK Classification of all Diagnoses.

Diagnosis	n (%) HIGH-RISK	LOW -RISK	Total
NMA	10 (18.5)	44 (81.5)	54
HNE	3 (33.3)	6 (66.7)	9
OLP	11 (35.5)	20 (64.5)	31
OLL	5 (33.3)	10 (66.7)	15
Leukoplakia no dysplasia	3 (23.1)	10 (76.9)	13
Dysplasia	6 (46.2)	7 (53.9)	13
TUGSE	2 (100.0)	0 (0.0)	2
OSCC	46 (86.8)	7 (13.2)	53

Twenty of 40 (50.0%) of NMA samples and 37/66 (56.0%) of the OPMD samples cultured positive for *Candida*. There was no difference in dSCORE between those samples with a positive *Candida* culture and those that did not for either group. There was no evidence of a difference in prevalence of HIGH-RISK classification associated with positive *Candida* culture (chi-square NMA p = 0.677; OPMD p = 0.861)

OSCC transformation correlates to increased dSCORE

One individual with a lesion diagnosed as a 'leukoplakia no dysplasia' developed an OSCC during the collection phase of this study and had a second sample collected at time of OSCC diagnosis. An initial incisional biopsy yielded a histopathological conclusion of squamous epithelial hyperplasia with mild atypia, insufficient for dysplasia associated with prominent inflammation **(**Fig. 4**A).** The patient was placed under periodic review. Six months later, the lesion changed in clinical appearance and underwent complete excision with a diagnosis of

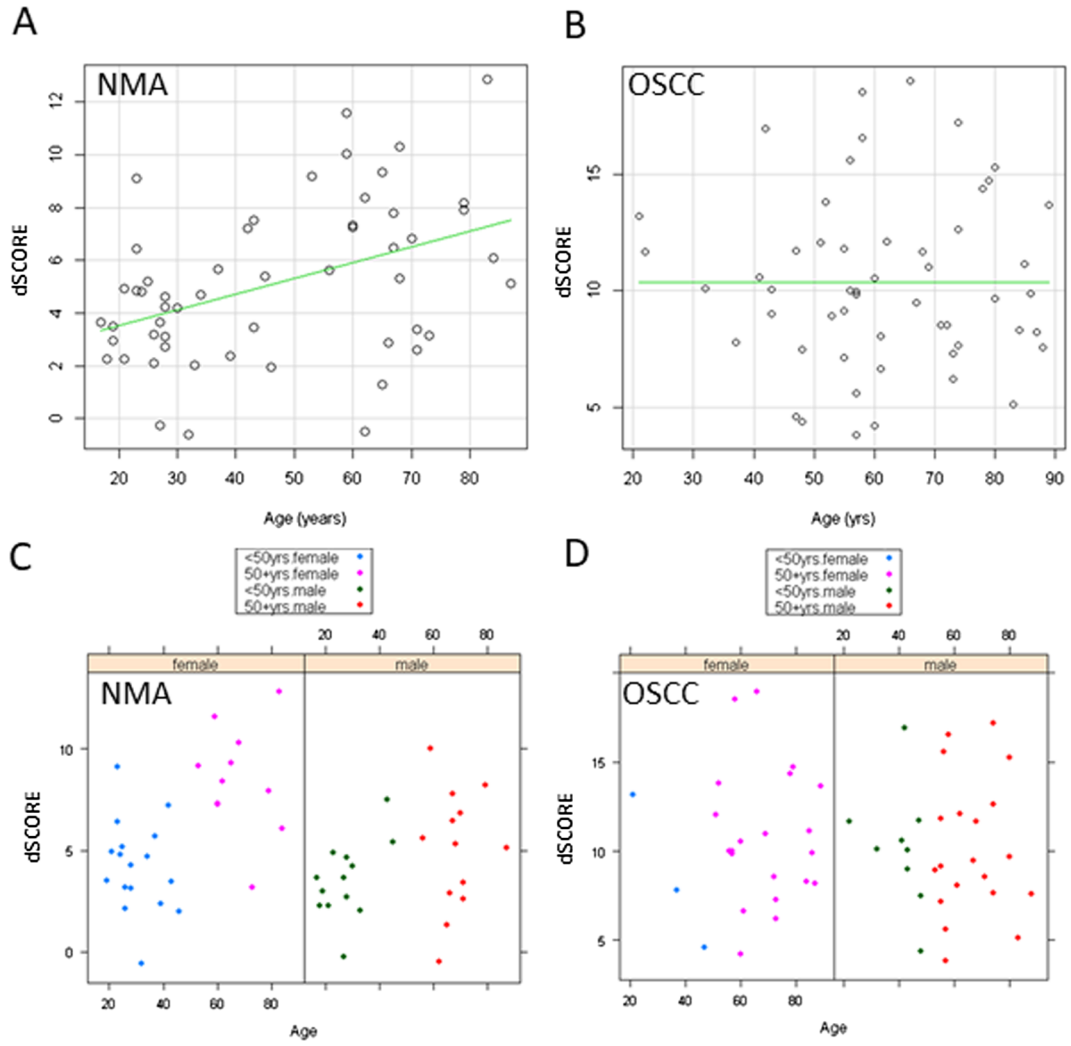

Fig. 3. (A) Age in years vs dSCORE of NMA (n = 54) with least squares line (green) shows a positive correlation (p = 0.001). (B) Age in years vs dSCORE of OSCC (n = 53) did not show a correlation (p = 0.996). (C) The dSCORE distribution in the NMA group demonstrated an independent effect of both older age (p = 0.001) and gender (p = 0.008) with older females. (D) dSCORE distribution of OSCC was not correlated with either age (p = 0.996) or gender (p = 0.935). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

T1N0M0 moderately differentiated squamous cell carcinoma **(**Fig. 4**B).** Analysis of her oral swirl samples found that her dSCOREs rose from 4.51 to 9.90 from initial biopsy to time of excision. The -RISK classification rose from LOW to HIGH **(**Fig. 4**C).** At follow-up 2.5 years later there was no sign of recurrence.

Discussion

This study clearly demonstrates consistent detection of a panel of oral cancer-associated microRNAs in individuals with a variety of demographics, oral and systemic condition, establishing a reproducible and consistent method for the isolation of sufficient RNA for microRNA analysis from oral swirls. Further, significant variation in a panel of selected microRNAs between patients with OSCC compared to individuals with NMA or OPMD was demonstrated.

The utility of identifying OSCC from NMA by quantifying 5 microRNA sourced from oral swirls was demonstrated by the dSCORE differentiating the NMA and OSCC with an AUC of 0.8676 and the -RISK category accuracy of 84.2%. A HIGH-RISK microRNA signature was prevalent in 86.8% of OSCC patients and 18.5% of a control group. The HIGH-RISK signature was not a phenomenon associated with late presentation or smoking and drinking habits.

The 5 microRNA studied here have not only been associated with OSCC [10] but also with other cancers. MicroRNA-24 has validated targets affecting cell proliferation, cell cycle arrest, apoptosis and differentiation. It has been implicated in squamous cell carcinoma and other cancers including lung, gastric, colorectal, breast, prostate, ovarian, pancreatic, leukemia [12]. Upregulation of miR-21 has been found in breast cancer tissue [13] and studied as a circulating biomarker of human carcinomas [14]. let-7c is known to be a repressor of the Wnt signaling pathway [15], and has been found to be downregulated in the tissue and serum of breast cancer patients [16] and significantly decreased in cervical intraepithelial lesions and cancer compared to normal cytology [17]. miR-100 targets multiple tumor-related genes such as mTOR, PI3K, AKT1, IGF1-R [18]. It may be that the HIGH-RISK signature represents common mutations associated with OSCC, however the differential expression in microRNA may also be present in other cancers or in tissues at increased risk of the development of cancer.

Salivary microRNAs have been of interest in screening for cancers distant to the oral cavity [19]. For example, it has been shown that salivary miR-21 was able to distinguish colorectal cancer patients with a sensitivity of 97% and specificity of 91% [20]. In Australia, it was estimated that the risk of being diagnosed with a cancer by age 85, in 2017, was 1 in 2 [21]. Our study did not physically assess for the presence of other malignancies. Determining the prevalence of this

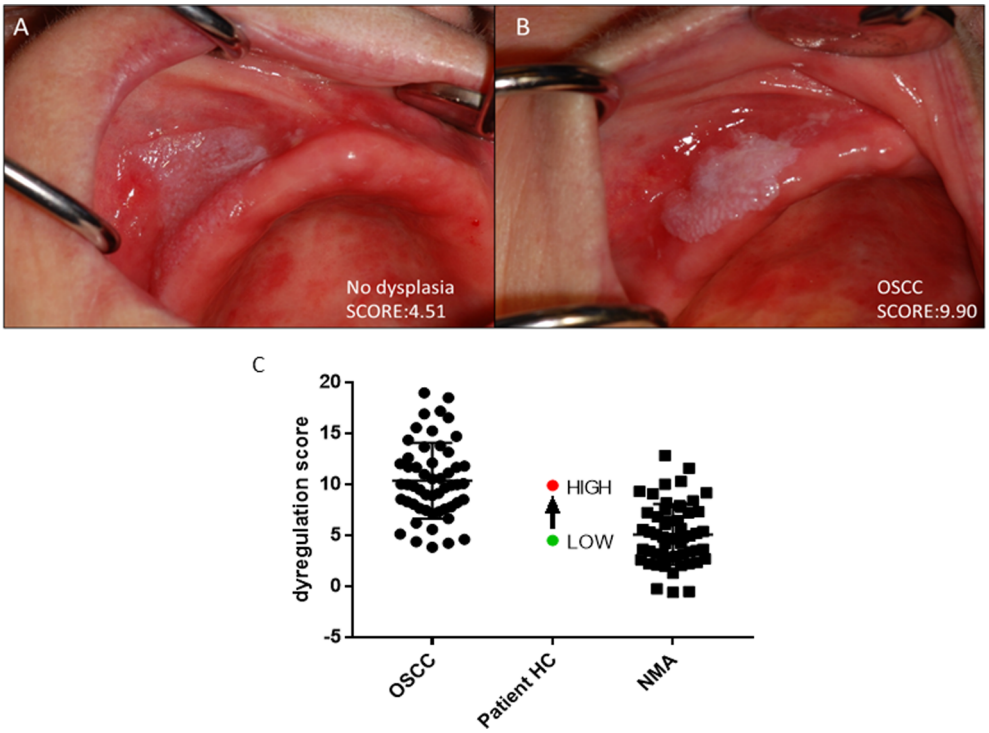

Fig. 4. Patient HC demonstrating (A) leukoplakia with no dysplasia and then (B) OSCC; (C) Change in dSCORE and -RISK category of Patient HC during transformation to OSCC with OSCC and NMA groups.

HIGH-RISK signatures in oral swirls of individuals with other malignancies distant to the oral cavity study would be of interest.

To examine the dSCORE and -RISK categories across the age spectrum, it was important to include individuals with a diverse age range within the NMA group, as older individuals are more likely to have acquired genetic insults, thus proliferation or cancer-associated mutations, than younger age groups. This was reflected by the higher prevalence of a HIGH-RISK signature in the over 50 yrs NMA group. It may be that age-specific reference ranges should be considered when considering dSCORE and -RISK, similar to prostate-specific antigen thresholds for the detection of prostate cancer [22]. Nevertheless, careful consideration of prioritizing sensitivity and specificity must be made.

The particularly high false positive rate in NMA females over 50 yrs (n = 11) may reflect that our cohort is underpowered for analyses within this subgroup. It is also possible that the positive rate is a true indication of risk of other malignancy, other than OSCC. However, a high false prevalence rate was not observed in the OPMD group relating to female gender, or age above 50 years or both. Further studies, particularly longitudinal studies, could provide further insight into the factors of female gender and older age.

The dSCORE and -RISK assessment algorithm was not affected by the presence of bacteria-mediated diseases of caries and periodontitis, or evidence of past bacterial virulence suggested by loss of teeth (reduced dentition and edentulism). Further, the score and prevalence of HIGH-RISK was not affected by the presence of culturable yeast within oral swirls. This supports that the selected workflow, including the EPIK Select assay has negligible cross-reactivity or background from non-human microRNA.

A major flaw of current diagnostic techniques for OPMDs is that these are unable to individuate patients that are at “at risk” of developing OSCC, although the presence of dysplasia is considered the best prognostic marker. In our study, the group with the lowest HIGH-RISK prevalence was the ‘leukoplakia with no dysplasia’ group, with a prevalence of 23.1% and the group with the highest HIGH-RISK prevalence was the ‘dysplasia’ group, with a prevalence of 46.2%. It was not surprising that the dysplasia group has the highest prevalence of the HIGH-RISK signature of all the OPMDs. Given that only a minority of OPMD progresses to OSCC, it is understandable that the mean dSCORE of the OPMD groups was not different from the NMA group, indicating that most OPMD are molecularly normal in terms of OSCC risk assessment. However, the mean dSCORE for the OSCC group was significantly higher than all other groups. Thus, the finding that the OSCC group had a distinctly higher mean dSCORE compared to the mean of the HNE and OPMD groups suggests clinical utility of the selected microRNA for early OSCC diagnosis. Furthermore, the correlation between salivary molecular markers of HIGH-/LOW-RISK and histopathological characteristics of OPMD (dysplasia/no dysplasia) shows potential for future use in risk stratification of OPMDs. In this regard, the case of malignant transformation during the study period clearly demonstrated the utility of oral swirls as a tool for longitudinal monitoring. A significant increase in dysregulation score and the emergence of HIGH risk where it was previously absent, could be an earliest indicator of OSCC. Serial oral swirls would provide a non-invasive sampling of the microRNA expression within the oral cavity. Their utility would be best as an adjunct to clinical and histopathological examination.

The selection of a limited number of microRNA’s in the present study allowed for an extensive analysis of the variation that exists across a large number of patients with diverse mucosal disease.

Conclusion

This study validates the use of oral swirls for analysis of microRNA using a simple, reproducible workflow. This is the first analysis of microRNA sourced from oral swirls from individuals with and without mucosal abnormalities, including OSCC and OPMDs. A HIGH-RISK signature was found to be accurate in indicating the presence of OSCC and exampled to parallel malignant transformation. Further longitudinal studies are warranted to assess this promising tool as a non-invasive indicator of molecular dysplasia.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.07.013.

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