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The assessment of the robustness of micro-RNAs from oral cytological scrapings

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ABSTRACT

Background: Sampling of suspect oral lesions in the general dental clinic may increase early carcinoma detection thus oral cancer survival rates. One means of lesion sampling that is an alternative to incisional biopsy is cytological scraping. MicroRNA alterations are also being explored as a means of diagnosing carcinoma as an alternative to histopathology. **Methods:** We obtained cytological scrapings using 10 strokes ("light") or 40 strokes ("heavy") from the buccal mucosa of one healthy subject using a dermatological curette. MicroRNA was isolated from oral cytological scrapings immediately or the scrapings were stored in buffer or RNA later, at 4°C, room temperature or 36°C, from 1-7 days prior to RNA isolation. All scrape comparisons and test conditions were conducted in triplicate. MicroRNAs were measured using qRT-PCR. **Results:** MicroRNAs can be obtained from cytological scrapings independent

of the number of strokes and can be measured using qRT-PCR after storage under all conditions tested.

Conclusion: MicroRNAs are robust to a wide range of storage conditions that bodes well for use of cytological scrapings to be of use in a clinical setting as a chair side sampling method for suspect oral lesions.

Introduction

MicroRNAs have been associated with almost all types of human malignancies including hematological¹ and solid tumors². Differential microRNA expression has been found in both oral premalignant and malignant cells³. Hence, there is a need for the investigation of microRNA expression in potentially malignant oral mucosal lesions as well as oral squamous cell carcinoma (OSCC) as deregulated microRNAs may be an early reliable marker for malignancy as well as a potential target for cancer prevention.

One of the factors limiting the OSCC detection rate is the reluctance of practitioners and patients to have repeat scalpel biopsies performed on suspect lesions⁴. Thus, although the developed method to assess oral cancer risk using microRNA is objective, it may not greatly improve OSCC detection rates as it provides no impetus for increased lesion sampling in the clinic.

Over the past years there has been renewed interest in oral cytology as an adjuvant clinical tool in the investigation of oral mucosal lesions. Several studies have shown encouraging results with oral brush cytology^{5,6}. However, there has been varying evidence cited with regard to the accuracy, sensitivity and specificity of OralCDx compared to routine scalpel biopsy, that has not supported the use of OralCDx in a routine manner⁷⁻⁹. Navone et al (2007) compared the histopathology of cells obtained using brush biopsy, exfoliation using a dermatologic curette to conventional scalpel biopsy¹⁰. The smears from the conventional cytology group were deemed inadequate in 12.4% of the samples (fewer than 30 well preserved cells from the intermediate or parabasal-basal epithelial layer) whereas, only 8.8% were deemed inadequate in the dermatologically curetted cytology group. A prospective study carried out on 164 patients with premalignant lesions (PML) comparing scalpel biopsy and micro-biopsy with a dermatological curette further supported the use of micro-biopsy⁶. Micro-biopsy diagnosis was in agreement with scalpel biopsy in 91.14% of the cases⁶. Its higher sensitivity levels than scalpel biopsy (97.65% vs. 85.88%) and its high negative predictive value (97.33%) suggest it to be an effective tool for diagnosis of PML⁶.

It has been demonstrated using comparison between FFPE and frozen tissues that microRNA are robust and preserved in scalpel biopsies^{2,11}. Tissue sampling via scalpel biopsy damages the layer of cells at the incision points but also provides a pool of cells within the tissue that would be undamaged by the action of

the scalpel. Thus, the RNA obtained from a scalpel biopsy would be a mixture of degraded and intact material. Cytological sampling using a curette may produce a greater proportion of damaged cells than biopsy which could impact comparative microRNA quality. To make a cytological scraping method viable for use in the general clinical setting the mode of sample storage and transport to a diagnostic laboratory also needs to be considered as the parameters of storage (transport) medium, temperature and time could also have an impact on the data obtained.

Ibberson and colleagues (2009) conducted an extensive study where microRNA abundance was assessed using microarray analysis or microRNA-specific real-time quantitative PCR (miQPCR) and tissue samples that were maintained on ice for defined periods of time (and thus slowly degraded) prior to RNA extraction¹². The conclusion of this study was that the loss of RNA integrity leads to unpredictable microRNA profiles for both array-based and miQPCR assays¹². However, scrutiny of these results show that even after degradation of RNA over a span of 2 hours, there is only a 2-4 unit CT change in microRNA levels¹². A much larger range of fold change has been observed for microRNA in the order of ten to hundred fold changes between the cancer samples and normal tissues^{13,14}. Thus RNA degradation that results in small relative changes may not significantly interfere with the profiling of microRNAs in cancers.

Nevertheless, the ability to extract RNA of maximal quality would aid in downstream analyses. Commercial products such as RNA Protect Saliva (Ambion) and RNA Later (Ambion) claim to prevent degradation of total RNA by immediately stabilizing RNA to preserve the gene expression profile¹⁵. Use of cytological scrapes as a source of cells would be of immense benefit to lesion diagnoses as it is a simple procedure that could be performed by a general dental practitioner during a patient visit when a suspect lesion is identified. However, prior to progressing to this step the suitability of such cytological scrapings for microRNA abundance detection needs to be assessed.

Any procedure which highlights neoplastic lesions should aid the clinician in detection of OSCCs. Toluidine blue dye has been used to enhance the visibility of lesions with increased nuclear density, thereby highlighting areas of cell proliferation¹⁶. Chemiluminescent light (Vizilite) has been advocated to enhance visualisation of oral mucosal white lesions and VELscope is used to distinguish abnormal tissue from normal via different levels of autofluorescence¹⁷. However, a major weakness of all these diagnostic aids is a lack of diagnostic correlation with biopsy findings¹⁸.

The aim of the present study was to assess the usefulness of cytological scrapings of oral mucosa obtained using a dermatological curette as a source of microRNA for downstream analysis. We also analysed the amount of degradation of microRNA during storage in various media, and with varying

temperature and time, prior to RNA extraction. We demonstrate that microRNA are robust and can be isolated from cells even after storage at 36°C for 7 days.

Materials and methods

Ethics approval was obtained from the Human Research Ethics Committee, The University of Melbourne, prior to commencement.

Sample collection

Oral epithelial scrapings were taken from the buccal mucosa of a subject using either 10 strokes (“light”) or 40 strokes (“heavy”) with a dermatological curette (Acuderm IncTM, USA). The scraped material was placed immediately in either 500 µl of lysis buffer from the mirVanaTM miRNA Detection Kit (Ambion®) or in 500 µl of RNA Protect Saliva (Qiagen IncTM). RNA in these scrapings was either extracted immediately, or stored for 24 hours, or 7 days. The samples were stored at either 4°C, room temperature, or 36°C. All scrapings and extractions were conducted in triplicates. To avoid inter-examiner discrepancies the scrapings were conducted by the one person and were taken from the same individual.

RNA extraction

Total RNA was extracted from the cytological samples using the mirVanaTM miRNA Detection Kit (Ambion®). Extracted RNA was treated with DNase according to the Turbo DNA-FreeTM Protocol (Applied Biosystems, Australia).

RNA amount and quality

Recovered RNA concentrations were measured using a Nanodrop 1000A spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and the quality assessed using an ExperionTM system (BioRad, Australia). The RNA Quality Indicator (RQI) value is calculated by the Experion software using an algorithm that compares the electrophoretic profiles of the 28S, 18S and pre-18S rRNA peaks. An RQI of 7 or more indicates acceptable mRNA quality¹⁹.

cDNA conversion

Reverse transcription (RT) of RNAs derived from the cytological scrapings was performed using the MegaplexTM Primers, Human Pools A (Applied Biosystems) with an additional pre-amplification step. Thirty nanograms of total nucleic acid was placed in each RT reaction. All samples were reverse transcribed and pre-amplified together with their respective negative control (no reverse transcription) and a PCR no template control. Human heart RNA (10 ng, Applied Biosystems) was used for positive reaction controls.

Quantitative PCR

To quantify the amount of microRNA extracted, quantitative PCR (qPCR) using TaqMan microRNA assays (Life Technologies) was utilised for miR-24b, miR-26 and miR-19b and the small RNA RNU 48. They were selected based on previous studies that have shown these microRNAs to be both abundant and ubiquitously produced^{20,21}. PCR was performed on all generated cDNA following the TaqMan Small RNA Assays Protocol using TaqMan® Universal PCR Master Mix 2X, With AmpErase® UNG (Life Technologies).

Data analysis

Thresholds were manually set for each microRNA or small RNA PCR at the point where the reaction was in its exponential phase but above any signal detected in negative control reactions. Cq values reported are the PCR cycle numbers at which the amplification fluorescence signal reached the chosen threshold. The Cq obtained for the real-time PCR results of were normalised using a Global Mean Normalisation (GMN) Strategy provided in the qbase PLUS software (Biogazelle 2008-2011) assuming normal distribution. Qbase PLUS provided calibrated normalised relative quantities (CNRQ) output for each sample.

Results

Detection of microRNA in cytological scrapings

QPCR of microRNAs from samples obtained using 40 strokes resulted in significantly lower Cqs for miR-19b and miR-26b ($P=0.003$ and $P=0.01$ respectively, unpaired t-test), reflecting higher abundance of target in these samples relative to use of 10 strokes (Table 1). In contrast there was no significant difference between the Cq for miR-24 from the light strokes (10) and heavy strokes (40).

There was a large variance in the Cqs for both light strokes ($SD= 4.16$) and heavy strokes ($SD= 5.39$) for RNU 48 compared to the Cq variations for the microRNAs with a trend towards lower Cq in those cells subjected to 10 rather than 40 strokes (Table 1).

Thus, these results indicated that use of 40 strokes during sample collection would produce better sensitivity (lower Cq) for detection of 2 of the 3 tested microRNAs, therefore for all subsequent studies, 40 scrapes were used when taking cytological scrapings.

Yield and quality of RNA harvested from differentially stored cytological scrapings

RNA yields from the exfoliated cells were variable, ranging from 5.1-32.5 ng/ μ l (Table 2) and the overall quality of the isolated total RNA, as shown by RQI values, also varied considerably. Samples stored in saliva protect tended to have higher RQI values, with scrapings extracted after being stored in saliva protect for 24 hours having a significantly higher RQI values ($P<0.05$) than scrapings stored in lysis buffer for 24 hours (Table 3). However, all samples produced poor quality RNA with RQI < 4 (Table 3).

Real-Time PCR

RT-PCR was conducted on cDNA produced using the various RNA extracts. Despite the poor RQI values, specific RT-PCR products were generated from all extracts indicating that microRNA and RNU 48 were recovered in all instances (Table 4). The lowest Cq values were obtained for miR-24 in samples stored in all of the conditions whilst miR-26b qPCRs had the highest Cq values in all storage conditions except in buffer for 7 days at 36°C (Table 4), where the Cq value for RNU 48 was highest. The variance in Cq across the different conditions ranged from 7-9 Cq, with the Cq value changes for each microRNA following the same trends. In general, lower microRNA Cq were obtained for samples stored in buffer rather than saliva protect. In contrast lower Cq were determined for small RNA RNU 48 when samples had been stored in saliva protect. Figure. 1 shows that the microRNA and RNU 48 tend to follow the same profile in Cq variation with storage condition, thus storage is having a reproducible effect on detection of the pool of target molecules.

GMN was used by qBase PLUS software to normalise the PCR Cq values to produce CNRQ values. No significant differences ($p>0.05$) in CNRQ of microRNAs or RNU 48 were observed between storing the samples at 4°C or above 4°C (room temperature or 36°C) (Table 5). The length of time the sample was stored also made no difference ($p>0.05$) to the mean CNRQ when comparing those samples stored for less than 7 days (RNA extracted immediately and at 24hours) with samples stored for 7 days (Table 4). However, two of the four PCR targets, showed significant ($p<0.05$) differences dependent upon storage media. RNU 48 had an 11 fold greater mean CNRQ when stored in saliva protect (CNRQ=5.57) rather than lysis buffer (mean CNRQ=0.49) (Table 5: $p=0.03$), while miR-26b showed the opposite with a higher mean CNRQ of 2.04 when the cytological scrapes were stored in lysis buffer rather than in saliva protect where CNRQ was 0.18 ($p=0.03$). No statistically significant differences were observed between the mean CNRQ for miR-19b and 24 when stored in different media (Table 5). Notably, the variances in CNRQ were generally large when compared to the mean CNRQ value.

Discussion

RNA integrity is the essential factor determining the accuracy of RT-qPCR measurements of messenger RNA transcripts, but there is little information on the relationship between RNA integrity and microRNA stability. Our findings are consistent with current research, conducted on renal and prostate cells, which show that microRNAs are robust even in heat degraded RNA preparations for which reliable mRNA detection is not possible²². Similarly, Patel et al. (2011) found that when whole saliva was stored in a RNA stabilizing agent and exposed to room temperature for 3 days, the levels of microRNA Let-7b remained constant²³. Our results demonstrate that RNA integrity does not prevent the obtaining of RT-qPCR measurements of microRNAs in oral cytological scrapings.

A large variance in the Cqs was observed for both light strokes (SD= 4.16) and heavy strokes (SD= 5.39) for RNU 48 (Table 1) indicating differential stability of RNU 48 relative to the microRNAs. In addition, large variances were observed in total RNA yield averages from cytological scrapings (Table 2) and CNRQ determined (Table 5) at any given storage condition. Reasons for this may relate to operator variability in obtaining cell samples, differential degradation during storage prior to extraction or a combination of both factors. A further factor in CNRQ variance could be variations in the subject at the time of sampling. For example, no controls were in place to assess for example when the subject last consumed food or beverages, brushed their teeth or used a mouth rinse, all of which may have impacted microRNA expression in the oral epithelium. Further research is required to fully assess these potential sources of variance. A robust normalization technique will be of critical importance in any cytological scraping method, particularly if it is to be used clinically, where operator and subject variances would be difficult to control.

The intactness or integrity that characterizes the quality of isolated RNA can be influenced by several factors, such as the RNA source, the sampling technique, the isolation procedures, and storage conditions²⁴⁻²⁶. Clinical samples in particular are subject to RNA degradation because of the often long interval between sample collection and their safe storage²⁴⁻²⁶. The low quality of the extracted total RNA did not prevent the ability to detect microRNA, even in degraded RNA samples. The overwhelming conclusion is that the microRNAs tested and RNU 48 are robust, even after storage at 36°C for 7 days, in either saliva protect or lysis buffer.

To be clinically relevant a lesion sampling method and biomarker would need to be robust to storage and transport conditions. The fact that time and temperature of storage of the cytological scrapings did not prevent the ability to extract and analyse microRNA indicates that cytological scrapings and microRNA analysis should be further tested for suitability for use in lesion analysis.

Comparison of cytological scraping storage methods indicates that for miR-26b it was better to use the lysis buffer as storage medium. The storage medium did not have any impact on the other microRNAs. The advantage of the above method is that samples can be stored directly in the lysis buffer and subsequently used in the first step of the RNA extraction protocol. This will not only save time and additional costs associated with purchasing Saliva Protect, but will also avoid the use of a reagent that requires removal before commencing total RNA extraction and thus reduce potential for error and increase technique sensitivity.

Overall, the lack of statistically supported variation amongst the Cq values found at the majority of the time points for the 3 microRNAs is indicative of the robustness of this assay for the analysis of microRNAs in cytological samples. Thus, in this proof of principle study, the robustness of microRNA, even after storage at 36°C for 7 days, indicates that the method presented here will be a potentially useful tool for analysis of cytological scrapings.

There may be two potential reasons for the observed robustness of microRNAs. The first is that the microRNAs are potentially encapsulated in exosomes and are therefore protected against degradation by salivary ribonucleases²⁷. Exosomes are cell-secreted vesicles of 30-100nm derived from the fusion of multi-vesicular bodies to plasma membranes and have been shown to be present in saliva²⁸. Exosomes may not only act to protect microRNAs, but also allow intercellular communication between cells in different anatomic sites²⁹. Finally, a second potential explanation for the observed stability of microRNAs is its structure. MicroRNAs form a protein complex with Argonaute-2 that has been shown to increase its stability in plasma and serum³⁰.

This was a pilot study investigating whether microRNAs can be extracted from oral cytological scrapings and to assess the robustness of microRNAs in these scrapes to storage under different conditions. The findings of our study show promising results and hence this methodology is now being extended onto a microRNA abundance study in a patient cohort with OLP, dysplasia and OSCC.

The development of a chair side diagnostic tool using a dermatological curette may have several benefits. Firstly, for non-compliant patients who are unlikely to return for a recall appointment or accept referral, this non-invasive test may aid in assessing risk of oral cancer. Additionally, it can be of use in patients with multiple oral lesions so as to decrease the need for multiple scalpel biopsies. Finally, for the long term management and review of patients who have either had a previous oral cancer or a persistent potentially malignant mucosal lesion, such a cytological tool would indicate the need and site for further investigation.

Conclusion

Despite varying storage mediums, storage conditions and time frames of storage of cytological scrapings, microRNAs can be obtained and measured using qPCR. Thus, the results presented here form the basis for the development of a simple robust, non-invasive diagnostic assay using microRNAs to assess the malignant potential of oral mucosal lesions.

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Table 1: Use of light or heavy curette strokes for sampling of buccal epithelia for detection of microRNA and RNU 48

Sample	Number of Strokes with the dermatological curette	PCR cycle at threshold			
		microRNA 19b	microRNA 24	microRNA 26b	RNU 48
1	10	23.74	21.31	28.12	19.13
2	10	23.6	17.69	27.37	19.77
3	10	23.63	20.08	28.19	26.63
	Mean of 10 strokes	23.66	19.69	27.89	21.84
	SD of 10 strokes	0.071	1.84	0.45	4.16
1	40	18.52	18.89	25.36	34.41
2	40	19.47	18.73	25.65	24.81
3	40	20.69	17.76	26.56	25.35
	Mean of 40 strokes	19.56	18.46	25.86	28.19
	SD of 40 strokes	1.09	0.61	0.63	5.39
	P value (10 strokes versus 40 strokes)	0.003	0.333	0.01	0.18

Table 2: Yield of RNA harvested from cytological scrapings stored in different conditions

TEMP	Yield averages+
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	(ng/ul)
Buffer, 7d, RT*	13.5 ± 2.7
SalivaProtect, 7d, RT	32.5 ± 23.5
Buffer, 7d, 36°C	12.3 ± 1.5
SalivaProtect, 7d, 36°C	9.8 ± 6.0
TIME	
Buffer, immediately placed	7.0 ± 2.1
Buffer, 7d, 4°C	15.5 ± 9.5
SalivaProtect, 7d, 4°C	17.2 ± 7.1
Buffer, 24hrs, 4°C	5.1 ± 0.6
SalivaProtect, 24hrs, 4°C	6.2 ± 2.8

*d=days ; RT= Room Temperature

+ yield average of 3 samples (n=3)

Table 3: Comparision of RQI values of scrapes stored in Lysis Buffer vs Saliva Protect

	Sample1 RQI	Sample2 RQI	Sample3 RQI	p value (Buffer vs saliva protect)
Buffer, 7d, RT*	2.1	1.9	2.2	
SalivaProtect, 7d, RT	3.9	4.2	2.7	0.07
Buffer, 7d, 36°C	1.8	1.9	1.9	
SalivaProtect, 7d, 36°C	2.5	2	ND**	0.68
Buffer, 7d, 4°C	3.4	3.8	3.1	

Salivaprotect, 7d, 4°C	2.3	4.2	4.6	0.75
Buffer, 24hrs, 4°C	3	3	2.8	
SalivaProtect, 24hrs, 4°C	3.4	3.3	ND	0.01

d=days ; RT= Room TemperatureND-Experion was unable to provide an RQI reading and instead gave a reading of “critically low anomaly.”*

Table 4: Real-time PCR Cq of microRNAs and RNU 48 detected in RNAs extracted from cytological scrapes stored in various conditions

Storage condition	MicroRNA							
	24		19B		26B		RNU48	
	Mean*		Mean*		Mean*		Mean*	
	Cq	SD	Cq	SD	Cq	SD	Cq	SD
Buffer_7d_RT	13.5	1.8	19.8	1.4	25.7	3.4	23.6	0.7
Buffer_7d_36deg	15.3	0.8	21.6	5.8	24.0	4.2	27.1	3.9
SalivaProtect_7d_RT	16.2	1.3	21.0	1.0	32.4	4.8	22.2	2.7
SalivaProtect_7d_36deg	18.6	3.4	26.5	3.0	32.8	2.7	20.1	2.6
Buffer_immediate extraction	20.8	3.4	21.0	3.6	28.4	4.9	28.2	5.4
Buffer_7days_4deg	14.7	2.6	19.2	1.3	25.4	4.9	24.9	4.4
SalivaProtect_7days_4deg	21.5	7.3	23.6	1.7	31.9	3.7	27.6	4.7
Buffer_24hrs_4deg	22.5	2.6	27.0	3.5	30.8	1.1	28.3	1.5
SalivaProtect_24hrs_4deg	18.1	3.8	25.2	2.8	30.5	3.3	24.5	2.6

*mean of 3 samples (n=3)

Figure 1: Detection of MicroRNAs in RNA Isolated from Cytological Scrapings Stored in Different Conditions. The Cq determined for each microRNA are represented by symbols (error bars omitted for visual clarity), n=3. *d=days ; RT= Room Temperature

Table 5: The effect of cytological scrape storage conditions on microRNA and small RNA abundance

Target	Storage condition	Mean CNRQ* (95% CI low : 95%CI high)	P value
miR-24	4°C	0.44 (0.10:1.98)	0.37
miR-24	>4°C	1.77 (0.41:7.71)	
miR-19b	4°C	1.64 (0.20:13.76)	0.49
miR-19b	>4°C	0.54 (0.04:7.42)	
RNU 48	4°C	0.99 (0.35:2.79)	0.49
RNU 48	>4°C	2.32 (0.08:68.04)	
miR-26b	4°C	1.01 (0.36:2.85)	0.49
miR-26b	>4°C	0.43 (0.02:12.65)	
miR-24	7 days	1.34 (0.43:4.22)	0.48
miR-24	<7 days	0.30 (0.02:4.41)	
RNU 48	7 days	1.66 (0.26:10.65)	0.86
RNU 48	<7 days	1.10 (0.10:12.10)	
miR-26b	7 days	0.60 (0.09:3.88)	0.86
miR-26b	<7 days	0.91 (0.08:10.06)	
miR-19b	7 days	1.09 (0.19:6.38)	0.86
miR-19b	<7 days	0.84 (0.01:134.62)	
RNU 48	Buffer	0.49 (0.26 : 0.93)	0.03
RNU 48	Saliva Protect	5.57 (0.94 : 33.12)	
miR-26b	Buffer	2.04 (1.08 : 3.86)	0.03
miR-26b	Saliva Protect	0.18 (0.03 : 1.07)	

miR-19b	Buffer	1.44 (0.21 :9.89)	0.70
miR-19b	Saliva Protect	0.64 (0.03 : 14.41)	
miR-24	Buffer	0.82 (0.12 :5.63)	0.99
miR-24	Saliva Protect	0.81 (0.16 :4.10)	

*mean of 3 samples (n=3)

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