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ORIGINAL ARTICLE

Oral swirl samples – a robust source of microRNA protected by extracellular vesicles

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BACKGROUND: MicroRNAs are small non-coding RNAs which are dysregulated in disease states, such as oral cancer. Extracellular vesicles, a potential source of microRNA, are found in saliva.

OBJECTIVE: To demonstrate that a quantifiable amount of microRNA can be isolated from oral swirl samples. Additionally, we hypothesized that extracellular vesicles may protect contained microRNA from degradation in these samples.

METHOD: A polyethylene glycol-based precipitation was used for extracellular vesicle enrichment of oral swirl samples. Comparison was made between samples treated with and without RNase. Further, samples from three subjects were exposed to a range of conditions over 7 days and assessed for presence of microRNA by reverse-transcription quantitative PCR. Extracellular vesicles from samples were identified under transmission electron microscopy.

RESULTS: An adequate quantity of microRNA for qPCR analysis was extractable from samples despite exposure to conditions under which degradation of RNA would be expected.

CONCLUSION: A technique was developed to isolate an adequate quantity of microRNA for analysis from oral swirl samples. Extracellular vesicle-associated microRNA may be protected from degradation. This technique moves towards chairside application of translational microRNA research in the field of oral cancer prognostics.

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Keywords: oral cancer; microRNA; extracellular vesicles; saliva

Introduction

The incidence of oral squamous cell carcinoma (OSCC) is increasing worldwide (Chaturvedi *et al*, 2013), and the 5-year survival has changed little over the past 30 years. The 5-year survival is profoundly diminished for patients presenting with late stage tumours compared with those presenting with small, localized disease (Sciubba, 2001). Furthermore, the effect of both the disease and treatment on appearance, speech, chewing, saliva and swallowing enormously impacts the patients' quality of life (Rogers *et al*, 2008).

The number of OSCCs that arise from precursor lesions is unknown; however, reports indicate that 16–62% of OSCCs may develop from a potentially malignant oral mucosal disorder (Lee *et al*, 2000). At present, there are no established markers or laboratory studies that can detect OSCC prior to a clinically measurable tumour (Mydlarz *et al*, 2010).

Extracellular vesicles (EVs) have been investigated as a non-invasive source of potential prognostic and diagnostic biomarkers (Kim *et al*, 2014). EVs are a complex group of vesicles which originate from distinct subcellular compartments (Torrano *et al*, 2016). Exosomes are a specific type of EV that are 30–150 nm in diameter and released from many cell types. Exosomes are distributed widely within various biofluids, have a protective stable lipid membrane, may contain lipids, proteins, messenger RNAs (mRNAs) and microRNAs (miRNAs) and can be taken up by neighbouring or distant cells as a means of intercellular communication (Pant *et al*, 2012; Sato-Kuwabara *et al*, 2015). In carcinogenesis, this may involve both cancer-promoting as well as cancer-protective cellular contents within both the tumour microenvironment and via the circulation to distant sites (Hannafon and Ding, 2013).

MicroRNAs are small non-coding RNAs, 18–22 nucleotides in length that mediate gene expression at the post-transcriptional level by degrading or repressing target mRNAs. Recent studies have showed that miRNAs are involved in numerous cellular processes, such as development, differentiation, proliferation, apoptosis, stress response, as well as cancer development (Mestdagh *et al*, 2008). Changes in miRNA expression have been associated with almost all types of human malignancies (Hui *et al*, 2009) and have been shown to be suitable for use as salivary biomarkers due to their ease of isolation and identification through quantitative PCR (Michael *et al*, 2010). The majority of miRNAs detectable in serum and saliva are reported to be within exosomes (Gallo *et al*, 2012).

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Salivary miRNAs have the potential to be both measurable biomarkers observable prior to clinically visible mucosal disease, as well as therapeutic targets.

The use of saliva for a clinically robust sampling method that can withstand time and temperature variability postcollection is attractive. However, presently whole saliva collection may take up to 30 min (Momen-Heravi *et al*, 2014), limiting its clinical adoption. Previous studies have used an oral rinse method of 10 ml of sterile saline or water to wet the patient's mouth for 30 s before collection in a sterile tube (Samaranayake *et al*, 1986; Borromeo *et al*, 1992). We have explored the use of an oral rinse method based upon these previous two methods that we will refer to as an oral swirl (OS).

The aim of the present study was to isolate EVs from OS samples for miRNA analysis and we hypothesized that extracellular vesicles may protect the contained miRNA from degradation. Thus, we assessed the robustness of this method for isolating miRNAs.

Methods

This study was approved by the Human Research Ethics Committee of the University of Melbourne (Ethics ID: 050900X).

OS sample collection

OS sample collection method involved requesting subjects to swirl 10 ml of sterile deionized water or PBS 'like a mouthwash' for 60 s without swallowing and then expectorate into a sterile plastic tube. All OS samples were then centrifuged for 4 min at 4°C at 4000 × ***g*** to pellet epithelial cells and large particles. The remaining cell-free swirl sample was stored at −20°C.

Enriching for extracellular vesicles and RNA extraction

Initially, enrichment for EVs of 1 ml of cell-free OS was undertaken using either 40% polyethylene glycol PEG 6000 (Sigma-Aldrich, Castle Hill, NSW) in PBS 0.5 M NaCl, or a proprietary EV precipitating reagent (Exoquick™, System Biosciences, Pablo Alto, CA, USA). One-ml aliquots of OS were mixed with either 1 ml of 40% PEG solution or 250 μl of Exoquick and placed overnight at 4°C. The samples were then centrifuged at 10 000 × ***g*** for 30 min before the supernatant was removed leaving approximately 100 μl of precipitant. RNA was extracted from the resultant 100 μl using the mirVana™ miRNA isolation kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and DNA removed using the Turbo DNA-free™ kit and measured by spectrophotometer (Nanodrop 2000c).

The PEG-based method was enhanced using 5 × 1 ml; aliquots of cell-free OS placed with 1 ml of 40% PEG overnight at 4°C in a 2.2-ml microcentrifuge tube. After centrifugation and discarding of the supernatant as indicated above, the remaining five aliquots of 100 μl were vortexed, pooled and centrifuged at 10 000 × ***g*** for 5 min and 400 μl of the supernatant removed. This resulted in a 100-μl precipitant from the original 5 × 1 ml aliquots. A pellet was not always visible. RNA was extracted from the resultant 100 μl solution as previously described.

Transmission electron microscopy (TEM) of OS samples. OS samples were prepared using the PEG-based technique previously described. A 2-μl aliquot was fixed in 10 μl of 1% glutaraldehyde in PBS overnight at 4°C, and 6 μl was absorbed onto glow-discharged 300-mesh heavy-duty carbon-coated formvar Cu grids (ProSciTech, Kirwan, QLD, Australia) for 5 min, and excess was blotted on filter paper (Whatman, Maidstone, UK). Grids were washed twice with MilliQ water and stained with 2.5% uranyl acetate. Images were taken on a Tecnai G2 F30 (FEI, Eindhoven, The Netherlands) transmission electron microscope operating at 300 kV (Bio21 Molecular Science and Biotechnology Institute, University of Melbourne) (Figure 1).

RNase treatment challenge

Three 10-ml OS samples were collected from one subject, pooled and split into 6 × 5 ml aliquots. All samples were enriched for EVs using the protocol described above resulting in 6 EV concentrated technical replicate samples. RNase A was added to three replicates to reach a final concentration of 100 ng μl^{-1} in 100 μl and incubated at 37°C for 10–15 min (analogous to protocols used to remove all RNA for DNA purification) to remove all free RNA (Cheng *et al*, 2014). RNA was then extracted from all samples using the mirVana™ miRNA isolation kit with one modification – the lysis buffer was substituted with TRI-Reagent™ (Sigma-Aldrich, Castle Hill, NSW, Australia). This substitution was intended to provide both inactivation of the RNase and lysis of EVs present. The remainder of the protocol was as per the mirVana™ kit protocol. The samples were then treated with Turbo DNA-free™ and measured by spectrophotometer. The samples were concentrated to 15 μl using a rotational vacuum concentrator and measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using a small RNA chip (Figure 2a,b).

RNA was reverse-transcribed using Megaplex™ RT Primers, Human Pool A v2.1 TaqMan® and the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) before pre-amplification using Megaplex™ PreAmp Primers Human Pools A and TaqMan® PreAmp Master Mix. Quantitative PCR was performed using TaqMan® Fast Universal PCR Master Mix no AmpErase® UNG and a small RNA-specific TaqMan® MGB probe for hsa-mir-24-3p (UGGCUCAGUUCAGCAGGAACAG) (Figure 2c). This miRNA was selected as it has been found to be a commonly isolated miRNAs in saliva samples (Park *et al*, 2009).

Thermal condition challenge

Seven 10-ml OS sample were collected on the same day within 1 h from three individuals with no oral mucosal abnormalities. Cell-free samples for each subject were subsequently divided into 14 × 5 ml aliquots and designated A1-A4, B1-B6 and C1-C4 (Figure 3). Samples A1 and A2 for each patient were immediately enriched for extracellular vesicles using the PEG-based method described above and RNA extracted using the mirVana™ miRNA isolation kit and DNA removed using the Turbo DNA-free™ kit and measured by spectrophotometer. For the next 7 days,

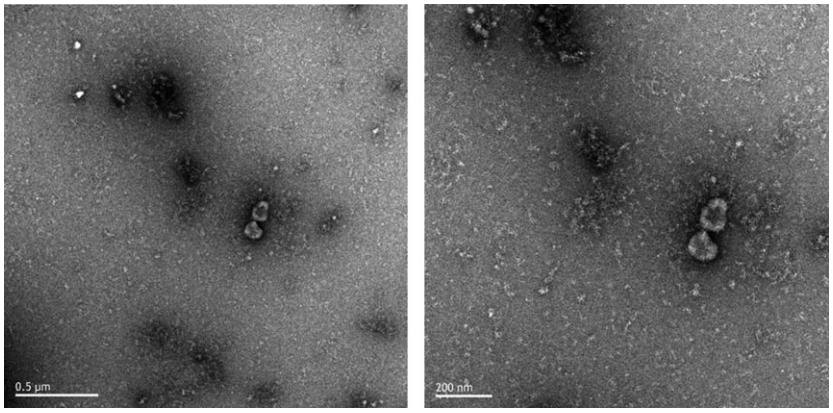

Figure 1 Extracellular vesicular structures visualized by TEM in OS samples. OS samples enriched for EVs were fixed and absorbed onto TEM grids. Extracellular vesicular structures with observable lipid bilayer membranes consistent in size with exosomes were visualized in OS samples

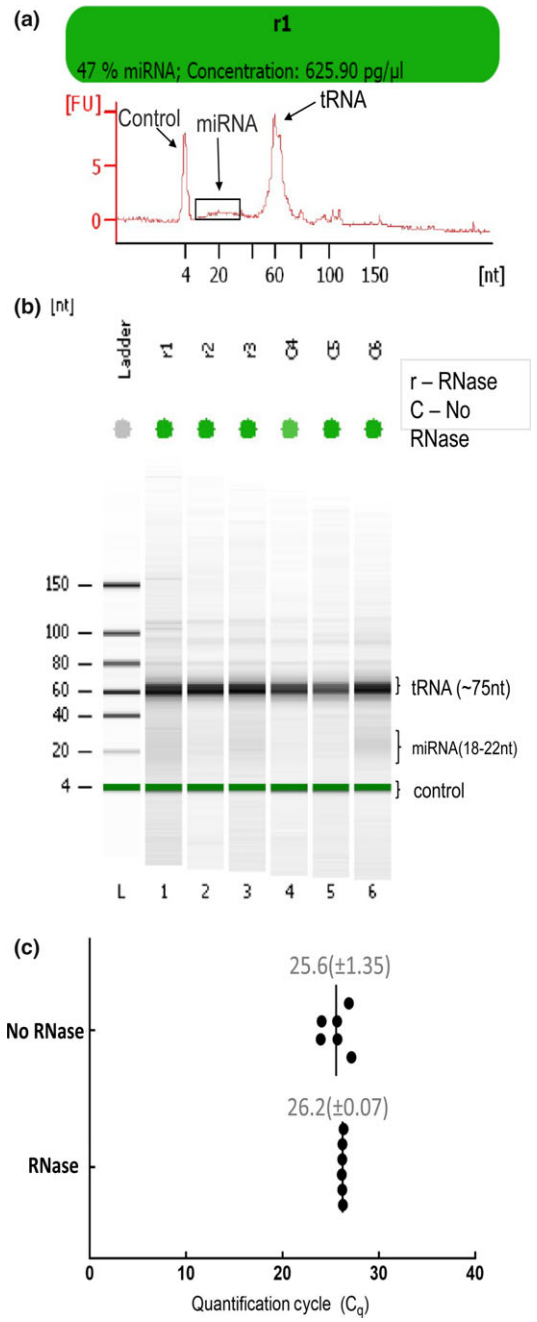

Figure 2 Preparations of OS enriched for EVs with and without exposure to RNase A before RNA extraction. Six aliquots of OS samples were concentrated for EVs. Samples r1-r3 were treated with RNase A before RNA was extracted. Samples C4-6 were not RNase A-treated. All samples were measured using a small RNA Agilent Bioanalyzer with electropherograms indicating the amount of microRNA, tRNA and small rRNA. (**a**) electropherogram for sample r1; (**b**) image generated from r1-3, C4-6 electropherograms; (**c**) C_q for hsa-mir-24 qPCR samples treated with RNase vs controls with mean and standard deviation [Colour figure can be viewed at wileyonlinelibrary.com]

samples A3 and A4 were placed at room temperature (20°C), samples B1 and B2 were placed at −20°C, B3 and B4 were placed at 4°C and B5 and B6 were incubated at 37°C. Samples C1 and C2 were exposed to four temperature shifts from -20°C to +20 and C3 and C4 from 4°C to +20 over the next 7 days. Samples A3-C4 were subsequently enriched for EVs and RNA extracted as above. The samples were then reverse-transcribed and quantitative PCR conducted as described previously.

Results

OS samples could easily be collected in a matter of minutes and multiple samples could be taken from one subject in one sitting. No statistical difference in RNA yield from EVs isolated from triplicate OS samples was found when comparing the use of deionized water (DW) with PBS (DW: $\mu = 5.37 \pm 1.87$ ng μl^{-1} s.d.; PBS $\mu = 3.97 \pm 0.20$ ng μl^{-1}; $P > 0.05$). Total RNA yield from EVs obtained using a 40% PEG solution protocol or the Exoquick™ protocol was similar with two matched samples enriched for EVs at the same time by both methods resulting in the first having 6.6 and 4.8 ng $\mu l^{-1}$and the second 4.4 and 5.1 ng μl^{-1}, respectively.

Extracellular vesicular structures with observable lipid bilayer membranes consistent in size with exosomes were visualized using transmission electron microscopy (Figure 1). Majority of vesicular structures observed had such size characteristics.

Avoidance of complete RNA degradation by RNase prior to EV isolation was tested in cell-free OS samples. Total RNA yield of samples treated with RNase ($\mu = 2.38 \pm 0.30$ ng μl^{-1}) was not statistically different to the non-RNase-treated samples ($\mu = 2.95 \pm 0.52$ ng μl^{-1}; $P > 0.05$). Figure 2a,b shows these same samples measured for small RNAs (size range 6–150 nucleotides). The mean percentage of miRNA of small RNA for RNase-treated samples (μ=40.3 ± 6.1%) was not statistically different to non-RNase-treated samples ($\mu = 24.3 \pm 20.3\%$; $P > 0.05$). The concentration of miRNA in small RNA for RNase-treated samples ($\mu = 493.5 \pm 147.5$ pg μl^{-1}) was additionally not statistically different to non-RNase-treated samples (μ=275.4 ± 366.7 pg μl^{-1}; $P > 0.05$). To confirm that miRNAs were isolated in these samples, real-time qPCR detection of hsa-mir-24 was undertaken. The result indicated that hsa-mir-24 abundance was not significantly different between preparations treated with RNase (Cq $\mu = 26.2 \pm 0.07$) and the non-RNAs-treated controls (Cq $\mu = 25.6 \pm 1.35$; $P > 0.05$) (Figure 2c). Thus, OS samples were protected from complete RNA degradation by RNase treatment prior to lysis of EVs. Interestingly, the RNase-treated samples had a much more consistent Cq for hsa-mir-24 with a standard Cq deviation of only 0.07 across six samples.

Protection of OS samples from RNA degradation was further assessed by exposing cell-free samples to thermal condition challenges for 7 days prior to EV enrichment and RNA extraction. OS samples were resistant to RNA degradation by thermal condition challenge even after 7 days. RNA yield varied between OS samples isolated from different subjects (Figure 4a), with some samples resulting in higher RNA yields than those immediately extracted, particularly those that were kept at room temperature or incubated at 37°C. Human versus microbial RNA was not differentiated at this step. The human microRNA hsa-mir-24 was detected by real-time PCR in samples exposed to all thermal conditions (Figure 4b). It can be seen that OS samples were protected from miRNA degradation by all thermal condition challenges, even after 7 days and repeated temperature changes. A slightly higher abundance (decrease in Cq values of RT-qPCR)

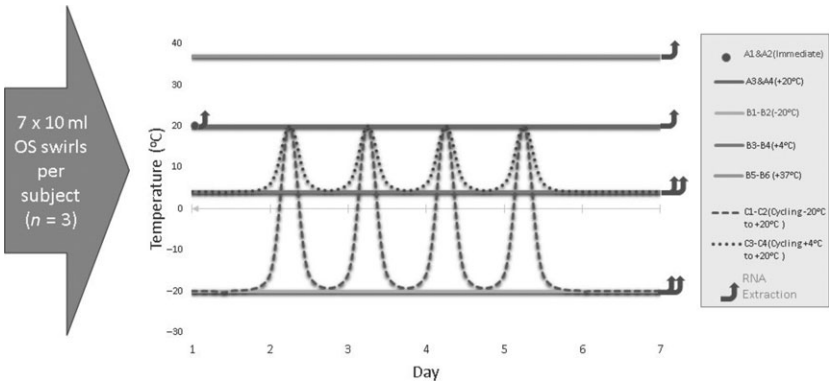

Figure 3 OS samples were exposed to four daily temperature shifts (−20 to 20°C and 4–20°C) or stored at −20, 4, 20 or 37°C over seven days prior to EV isolation and RNA extraction

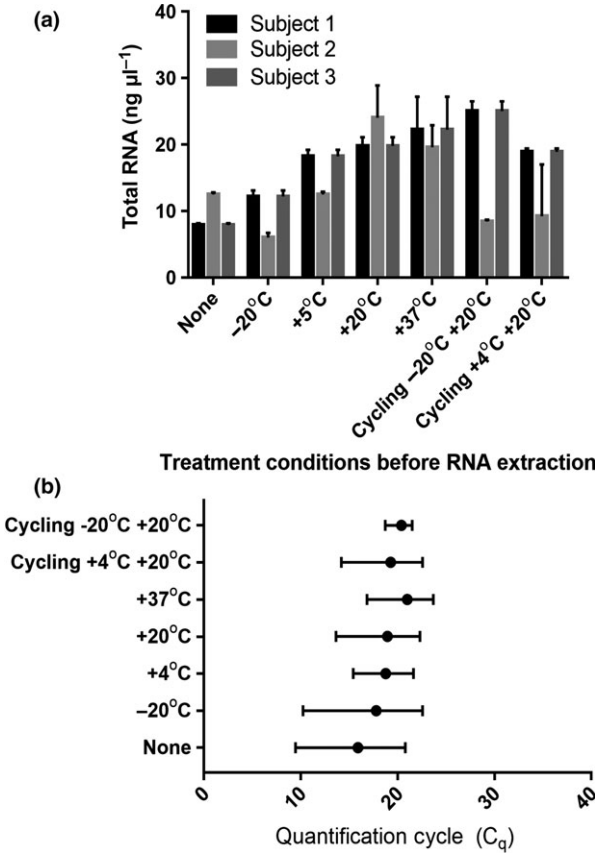

Figure 4 RNA extracted from OS samples exposed to temperature challenges over 7 days. OS samples were exposed to four daily temperature shifts (−20 to 20°C and 4–20°C) or stored at −20, 4, 20 or 37°C over 7 days prior to EV isolation and RNA extraction. (**a**) Mean and range of total RNA content measured using a spectrophotometer; (**b**) microRNA has-mir-24 content assessed using RT-qPCR

can be seen when samples were either immediately extracted or kept frozen at −20°C compared with those incubated at a higher temperature; however, no statistically significant differences were observed ($P > 0.05$).

Discussion

We have developed a simple and robust method for isolating microRNA from cell-free OS samples by enriching these samples for EVs. The isolated EV-associated microRNA have been shown to be protected from degradation and the microRNA is in sufficient quantity for assessment of variations that may be of clinical utility. This clearly demonstrates a simple and robust technique that is a significant step towards chairside application of translational microRNA research in the field of oral cancer prognostics.

The introduction of a clinical test requires balance between clinical benefit, and the time and additional skill required to administer it. Saliva, and saliva-based clinical collection, is non-invasive and easy, and thus attractive. Overcoming the time limitation of collecting whole saliva and establishing a 'minimum required sample' for appropriate downstream analysis is desirable. Use of OS collection may provide a convenient sample source for biomarker analysis.

EVs have been shown to provide a protective and enriched source of microRNA compared with intracellular and cell-free blood (Cheng *et al*, 2014). The present study clearly demonstrates that they are protective of microRNA in oral swirls. Methods of OS handling and EV isolation developed here indicate that the use of OS for microRNA isolation for biomarker analysis will be a viable approach.

Novel compounded products for EV isolation can be costly. An easy to compose mixture which could be created and used at a low cost in any laboratory would encourage widespread availability and use of a test. The use of polyethylene glycol for viral and protein precipitation is an established methodology (Ingham, 1984; Lewis and Metcalf, 1988). EV precipitation using PEG is simple, cost-effective and avoids time-consuming ultracentrifugation. The limitation of PEG is that it may allow for non-specific precipitation. This is particularly relevant to salivary samples as these are expected to contain a large amount of debris, including viral, bacterial and fungal elements, in addition to salivary components such as proteins. Such 'contamination' was clearly demonstrated when visualizing these samples with electron microscopy. Furthermore, exposure of these samples to temperature challenge for 7 days showed an increase in nucleic acids for those samples left at room temperature or incubated at 37°C. This additional nucleic acid was likely bacterial or fungal, and as such it is therefore important that downstream analyses utilize tests highly specific for human biomarkers. In the present study, we examined microRNA

using human-specific primers, confirming not only their presence, but that microRNA has been protected from degradation during thermal challenge. Thus, isolating assessable miRNA in the present study from OS samples after placement in various thermal conditions for 7 days is supportive that miRNA protection by EVs is a robust method that is temporally stable.

It would be expected that RNA present in salivary swirls would be degraded by RNase A unless there exists a barrier of protection within OS samples. Isolation of comparable quantities of RNA from samples treated and not treated with RNase suggests that the majority of miRNA in the OS samples were contained within EVs and that these EVs were protective of RNase degradation. The improved consistency in hsa-mir-24 qPCR Cq following RNase treatment may well be due to the degradation of extravesicular mRNA and microRNA, likely derived from lysed epithelial cells or free salivary RNA in the OS, thus resulting in the sampling of RNA derived solely from EVs. Further studies are required, but it would appear that the consistency of miRNA isolation using the EV isolation method developed in the present study shows significant promise for accurately and reproducibly measuring comparative abundances of OS-derived microRNAs. Such a method is likely critical in the assessment of samples from patients with no mucosal abnormalities, those with inflammatory mucosal disease and those with oral cancer. It may well be that EV contained miRNA variation is observable in patients with OSCC prior to a lesion becoming clearly visible to the clinician.

Our study has demonstrated that utilizing a simple, non-invasive OS method allowed sufficient miRNA to be isolated for analysis. The method requires validation in individuals with a range of salivary flow rates and different oral mucosal diseases. Moreover, there is clearly the need to extend this OS method of collection, EV enrichment and miRNA extraction to a prospective study and subsequently multicenter studies, so as to explore the usefulness of salivary EV miRNA variation for the early detection of OSCC.

Conclusion

The presence of exosome-sized extracellular vesicles has been demonstrated in OS samples. Assessable miRNA was able to be isolated in OS samples despite the sample being exposed to conditions expected to cause RNA degradation. EVs in OS samples may provide a stable source of miRNA for use as a biomarker, shielded from degradation. EV-enriched OS sampling may be the link to advance the clinical translation of miRNA in the prediction of OSCC development in individuals with and without potentially malignant oral mucosal disorders.

Acknowledgements

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Conflict of interest

None Declared.

Author contributions

T. Yap was primary investigator and was involved in the whole study design, data anaysis and draft of the paper. L. Vella performed TEM analysis, and review of the paper. C. Seers was a supervisor and was involved in study design, data analysis and draft and review of the paper. A. Nastri was involved in review of the paper. E. Reynolds was involved in review of the paper. N. Cirillo was involved in study design and review of the paper. M. McCullough was primary supervisors and was involved in study design, data analysis, draft and review of the paper.

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