Original Research Article

Differential miRNA expression in oral cancer oncosomes: a pilot study.

Abstract

Aims: Exosomes are small membranous secreted vesicles (30-120 nm) believed to function as intercellular messengers delivering their cargo of RNA and protein to target cells. While many cells secrete exosomes, cancer cells have been found to produce higher numbers of exosomes than normal cells. Cancer specific exosomes, also termed oncosomes, transport intercellular bioactive molecules including proteins, lipids, and microRNAs (miRNA), the latter of which are discarded into the extracellular environment via exosomes. These bioactive molecules can modulate oral squamous cell carcinomas (OSCC) disease progression *in vivo*. To date, only one study had demonstrated the secretion of oncosomes from cultured OSCC cells, therefore the objective of this study is to determine if intact oncosomes can be isolated from oral cancer cells.

Study design: This is an observational laboratory-based study of human oral cancer cell cultures.

Place and Duration of Study: This study was conducted in the Department of Biomedical Sciences at the University of Nevada, Las Vegas – School of Dental Medicine between May 2016 and May 2017.

Methodology: Using a reagent that binds water and forces less-soluble lipid vesicles out of solution, oncosomes from oral cancer cell cultures (SCC4, SCC9, SCC15, SCC25 and CAL27) were collected by low-speed centrifugation. qRT-PCR was performed on RNA isolated from the culture-derived oncosomes for miR-21, miR-365, miR-155 and miR-133a1; all previously identified from cancers of other tissues.

Results: Normal, non-cancerous HGF-1 (human gingival fibroblasts) had low (almost) undetectable expression of miR-21, -133, -155, and -365. Oral cancer cell lines (SCC4, SCC9, SCC15, SCC25 and CAL27) had moderate to high expression of at least one microRNA – although this varied significantly by cell line.

Conclusion: Exosomes can be successfully isolated from OSCC conditioned media and miRNAs are detectable through TaqMan microRNA assays, with unique characteristic expression of the miRNAs in the cell lines examined. Although more investigation is needed, potential correlations between miRNA levels and proliferation rates were also observed.

Keywords: oral cancer, oncosomes, microRNA

Abbreviations: Ribonucleic acid (RNA), microRNA (miRNA), oral squamous cell carcinoma (OSCC), Squamous cell carcinoma (SCC), American Type Culture Collection (ATCC), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS). Reverse transcription (RT), Quantitative polymerase chain reaction (qPCR), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), deoxyribonucleotide triphosphate (dNTP), dimethyl sulfoxide (DMSO),

1. Introduction

Exosomes are small membranous secreted vesicles (30-120 nm) believed to function as intercellular messengers delivering their cargo of ribonucleic acids (RNA) and protein molecules to target cells. Exosomes are secreted by numerous types of cells in culture and are found in abundance in body fluids including blood, saliva, urine, amniotic fluid, cerebrospinal fluid, nasal secretions, and breast milk [1]. While many cells secrete exosomes, cancer cells have been found to produce higher numbers of exosomes than normal cells [2]. Cancer specific exosomes, also termed oncosomes, transport bioactive molecules including proteins, lipids, and microRNAs (miRNA), which are transferred into the extracellular environment via exosomes.

These bioactive molecules have been demonstrated to modulate oral squamous cell carcinomas (OSCC) disease progression *in vivo* [3]. In fact, oncosome miRNA, which are small non-coding RNA only containing on average 22 nucleotides, are now known to be fundamental regulators of both mRNA and protein expression [1,2]. However, the isolation of intact exosomes is traditionally accomplished using ultracentrifugation, a method that is difficult and requires specialized equipment [4]. One objective of this research project was to determine if intact oncosomes could be isolated from OSCC cells using a reagent that binds water molecules and forces less-soluble lipid vesicles out of solution allowing the oncosomes to be collected by low-speed centrifugation.

To determine if this procedure was possible, it was necessary to focus on a few specific miRNAs for this initial pilot study. While over 750 miRNAs have been found in exosomes, the expression of four specific miRNAs: miR-365, miR-21, miR-155, and miR-133a-1 have been extracted from multiple types of cancers, including oral cancers [5]. These miRNAs may be responsible for differential induction of mRNA in target cells, including both up- and down-regulation of mRNA transcription and translation.

More specifically, one of the most widespread and over-expressed miRNAs in human malignancies has been identified as miR-21, an inhibitor of PTEN and pro-apoptotic genes, thereby allowing cell survival [6]. In addition, although miR-155 plays a vital role in various physiological and pathological processes such as hematopoietic lineage differentiation, immunity, inflammation, and cardiovascular diseases - some evidence also demonstrates miR-155 as overexpressed in a variety of malignant tumors as well as having a role in the spread of viral infections [7]. In contrast, miR-133 is expressed in muscle tissue and appears to repress the expression of non-muscle genes as well as SRF transcription factor and cyclin D2, which may modulate, in part, cell cycle progression [8]. Finally, although the role of miR-365 in cancer cells is still controversial, recent evidence may suggest an alternative, protective role that may inhibit growth, invasion and metastasis of malignant melanomas – although these functions are not well understood [9].

Squamous cell carcinomas (SCC) of the tongue represent a significant proportion of solid cancers that are diagnosed in late (metastasis forming) stages, therefore the ability to identify any prognostic indicators such as the production and presence of specific miRNA-containing oral oncosomes detectable from saliva could greatly improve detection and screening measures and help to improve clinical outcomes.

2. Methodology

Cell Culture

Human oral squamous cell carcinoma lines, SCC-4 (CRL-1624), SCC-9 (CRL-1629), SCC-15 (CRL-1623), SCC-25 (CRL-1628), and CAL-27 (CRL-2095) were obtained from the American Type Culture Collection (ATCC: Manassas, VA). The normal human gingival fibroblast cell line HGF-1 (CRL-2014) was also obtained from ATCC and used as a normal control for comparison. All cell lines were maintained in Dulbecco's Modified Eagles' Medium (Hyclone, Logan, UT) containing 4.0 mM L-glutamine, 4.5 g/L glucose, and 110 mg/L sodium pyruvate. Medium was supplemented with penicillin (100 units/ml) and

streptomycin (100 ug/ml) both from Hyclone and 10% fetal bovine serum (FBS). Cells were cultured in 75 cm2 or 25 cm2 BD Falcon tissue-culture treated flasks (Bedford, MA) and grown at 37°C and 5% CO2 in humidified incubators. Cell cultures were passaged during log phase growth at approximately 80% confluence at a sub-cultivation ratio of 1:3.

Exosome Isolation

Intact exosomes were isolated from cell culture. Cells were cultured in T75 flasks and switched to medium containing exosome depleted FBS and incubated for 24 hours before harvest. The conditioned medium was centrifuged at 2000 x g for 30 minutes to remove cells and debris. The medium was decanted and mixed with 0.5 volumes of Total Exosome Isolation reagent (Life Technology) before refrigerating overnight. To pellet the exosomes, the conditioned medium was centrifuged at 10,000 x g for one hour at 4℃. The pellet was resuspended in 200µL of 1X Phosphate Buffered Saline (PBS).

Exosome RNA Extraction

One volume of 2X Denaturing solution (Life Technology) was added to the resuspended exosomes before incubated on ice for 5 minutes. One volume of Acid-Phenol: Chloroform was added and the solution centrifuged at 4℃ for 5 minutes at 10 x g. The upper aqueous phase was carefully removed and 1.25 volumes of 100% ethanol added. The sample was pipetted into a filter and centrifuged at 10 x g for 15 seconds. The filter was then washed with miRNA Wash Solution 1 (Life technology) and centrifuged for 15 seconds, this same process was repeated twice more with Wash Solution 2/3. The filter apparatus was then centrifuged on its own for 15 seconds to remove any residual fluid from the filter. The filter was then placed into a new collection tube and 100 µL of heated RNase water was applied to the filter before being centrifuged for 30 seconds. The raw RNA was contained in the flow-through.

TaqMan MicroRNA Assays

Reverse Transcriptions (RT) carried out were 15 µL reactions with a master mix consisting of 100mM deoxyribonucleotide triphosphate (dNTPs), 10X reverse Transcription Buffer, RNase Inhibitor, and MultiScribe Reverse Transcriptase added to that mixture was 3 µL of the miR specific primer. The thermal cycler was set to 16℃ for 30 minutes, 42℃ for another 30 minutes, then 85℃ for five minutes before cooling to 4℃. Next a quantitative polymerase chain reaction (qPCR) was carried out using 20 µL reactions consisting of 20x TaqMan Small RNA assay, TaqMan Universal PCR Master Mix II and 1.33 µL of the corresponding product from the RT-PCR reaction. The thermal conditions for the reactions were 50℃ for 2

minutes, 95℃ for 10 minutes, then 40 cycles of 95℃ for 15 seconds and 60℃ for 60 seconds. Standard curves were made doing a five-fold serial dilution of CAL-27 cDNA for miR-365, miR-21 and miR-155 while standard curves for miR-133a was done with SCC-9 cDNA.

Thermo Scientific Verso 1-Step RT-PCR. Reverse transcription and PCR were carried out together in a single 25 µL reaction containing 2.5 µL of exosomal RNA, the desired forward and reverse primers, RT enhancer, 2X RT-PCR buffer, and versa reverse transcriptase enzyme. Thermal conditions for the reactions followed 50℃ for 30 minutes, 95℃ for 2 minutes then 35 cycles of 95℃ for 20 seconds, 55℃ for 30 seconds and a 72℃ extension for 1 minute.

Vybrant MTT Cell Proliferation Assay

Cells were plated at a density of 1 x 10^4 cells/well in triplicate on a 96 well plate. One plate was incubated for 24 hours, the second for 48 hours, and the third for 72 hours. When harvesting, first, all medium was replaced with 100 µL of fresh, phenol red-free medium. Then, 10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT stock solution was added to each well. A negative control was added to 6 wells containing just the medium alone. The plate was incubated at 37℃ for 4 hours. After incubation, all but 25 µL of medium was removed from the cells before 50 µL of dimethyl sulfoxide (DMSO) was added to each well. The plate was then incubated again for 10 minutes before the absorbance reading at 540nm.

3. Results

All cell lines were incubated in complete media supplemented with exosome-depleted FBS, which resulted in visible exosome precipitation pellets for exosomal RNA isolation and subsequent one-step RT-PCR experiments (Figure 1). TaqMan MicroRNA Assays for miR-365, miR-21, miR-155, and miR-133 were performed and standard curves for each of the miRNAs were generated from cDNAs , which allowed relative quantitation of each of the target miRNAs.

Normal HGF cells contained very low levels of the four target miRNAs with cycle thresh-holds exceeding 30 cycles. The OSCC cell line, CAL-27 demonstrated moderate levels of all the examined miRNAs with the exception of miR-133 which was 8-fold less abundant. The expression profile of the miRNAs in the OSCC cell line, SCC-9, was remarkably different

153 demonstrating low levels of miR-365, miR-155, and miR-21 with moderate levels of miR-
154 133.

155 Both SCC-15 and SCC-25 contained approximately 4-fold more miR-21 than the other
156 miRNAs examined, although most levels were comparatively low. SCC-4 cells contained the
157 highest levels of the miRNAs examined with very high levels of miR-365 and miR-21 and
158 moderate expression of miR-155 and miR-133.

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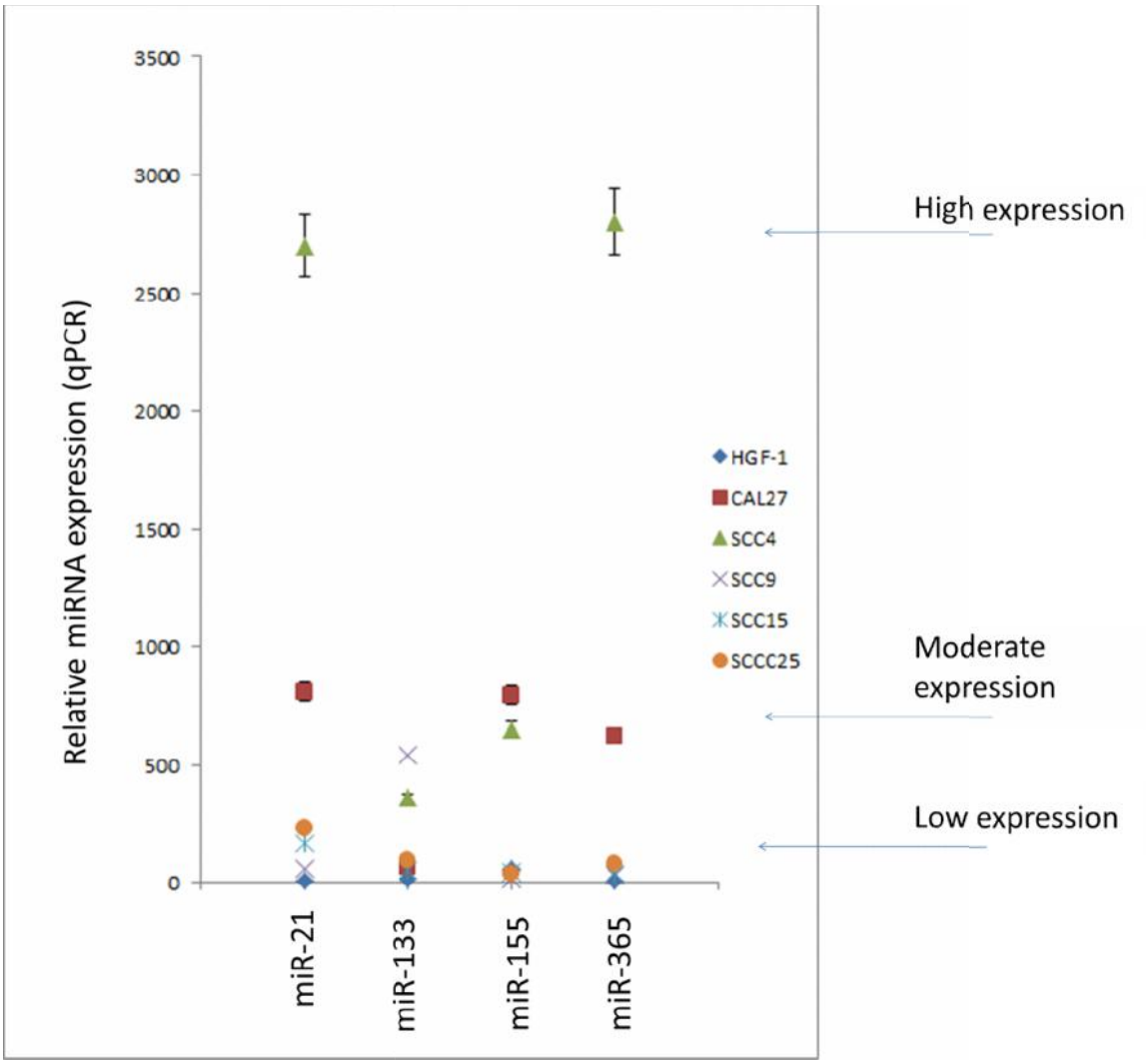

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161 Figure 1. Relative miRNA expression of oral cancers. Quantitative qPCR from exosome-
162 derived cDNAs allowed relative quantitation of miR-21, miR-133, miR-155 and miR-365.
163 Low-level expression was observed in HGF-1 (normal) cells, with differential expression
164 observed among the oral cancer cell lines. High-level expression of miR-21 and miR-365 was

observed among SCC-4 cells, which exhibited moderate expression of miR-133 and miR-155. CAL-27 cells exhibited moderate expression of all microRNAs except miR-133. Moderate expression of miR-133 was observed among SCC-9 cells.

These quantitative data derived from the qPCR were compiled and microRNA levels categorized with the relative-fold expression (RFE) as Low (RFE < 100), Moderate (RFE =100-1000), and High (RFE > 1000) (Table 1). These data demonstrated that each oral cancer cell line expressed moderate or high levels of at least one microRNA, with four of the five expressing moderate or high levels of miR-21. Two oral cancers (SCC-4, SCC-9) expressed moderate levels of miR-133, while two cell lines (SCC-4, CAL-27) expressed moderate levels of miR-155. Finally, two oral cancers expressed moderate or high levels of miR-365 (SCC-4, CAL-27).

Table 1. Quantification of differential miRNA-containing oral cancer exosomes.

	miR-21	**miR-133**	**miR-155**	**miR-365**
HGF-1 (normal)	LOW (RFE = 9)	LOW (RFE=13)	LOW (RFE=57)	LOW (RFE=9)
SCC-25	**MOD** (RFE=230)	LOW (RFE=10)	LOW (RFE=40)	LOW (RFE=75)
SCC-15	**MOD** (RFE=160)	LOW (RFE=50)	LOW (RFE=30)	LOW (RFE=75)
SCC-9	LOW (RFE=40)	**MOD** (RFE=500)	LOW (RFE=30)	LOW (RFE=20)
SCC-4	**HIGH** (RFE=2800)	**MOD** (RFE=400)	**MOD** (RFE=600)	**HIGH** (RFE=2800)
CAL-27	**MOD** (RFE=800)	LOW (RFE=30)	**MOD** (RFE=800)	**MOD** (RFE=650)

In addition, cellular proliferation was measured for all cell lines to determine if any associations might exist between growth rate and miRNA expression (Figure 2). These data revealed that the cell lines exhibiting the highest RFE levels of miRNAs were also the most rapidly dividing cell lines (CAL-27, SCC-4). Moreover, the cell line with the slowest relative growth overall (HGF-1) was also the only culture that exhibited low miRNA expression for

all the microRNAs examined. The other cell lines with moderate expression (SCC-9, SCC25) were also found to exhibit more modest growth in comparison with the CAL-27 and SCC-4 cell lines.

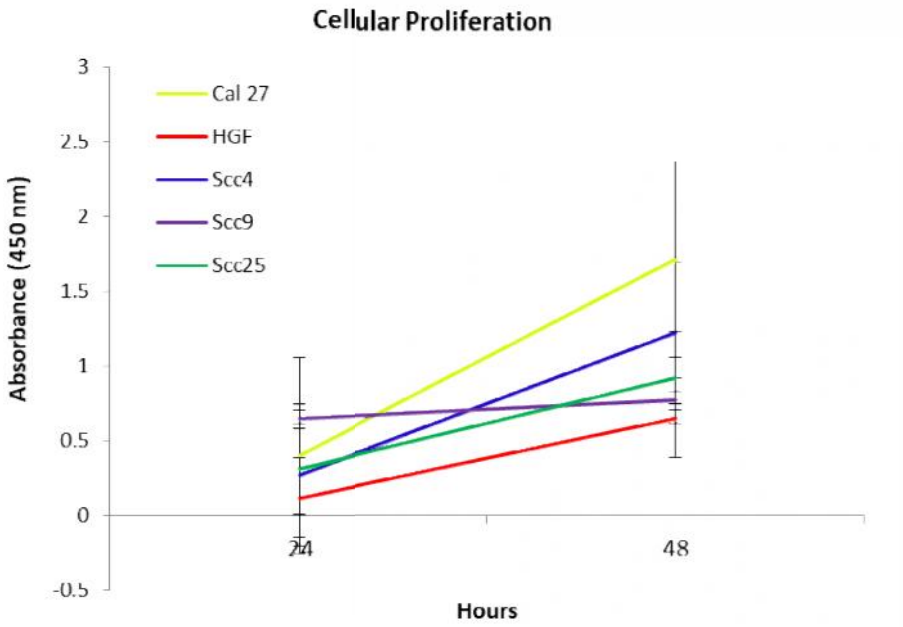

Figure 2. Proliferation results of HGF-1, CAL-27, SCC-4, SCC-9, and SCC-25. Examination of cellular growth demonstrated some cells with rapid growth (CAL-27, SCC-4), some with more moderate growth (SCC-9, SCC-25), with the slowest growth observed among the normal human gingival fibroblast (HGF-1).

4. Discussion

MicroRNAs hold great promise as potential biomarker and prognostic indicators for various cancers [11,12]. In addition, a better understanding of the molecular basis of miRNA expression as they relate to tumorigenesis and metastatic potential is necessary to advance the potential of miRNAs for improving clinical outcomes and for use as anti-cancer agents. [13,14]

Of the miRNAs examined in this study, miR-21 has been the most extensively investigated to date. miR-21 acts as an oncogene by decreasing expression of PTEN ultimately promoting

cell cycle progression and proliferation and inhibiting apoptosis via an AKT-dependent pathway [15,16]. Many independent studies have demonstrated overexpression of miR-21 in biopsies of head and neck tumors [10,17,18]. In addition, some studies have reported that miR-21 expression may be an independent prognostic factor indicating poor patient survival in tongue squamous cell carcinomas [19-21]. In the present study, miR-21 was found to be overexpressed in four of the five OSCC cell lines examined.

Interestingly, few studies have evaluated miR-133 in oral or head and neck cancers [10,22]. These studies found miR-133 levels to be downregulated in recurrent head and neck tumors but may be otherwise highly expressed. This study demonstrated low RFE for miR-133 in three of the five oral cancers evaluated, with CAL-27 (the fastest growing) exhibiting among the lowest expression. However, the low levels observed among the normal and slower growing cancers may not directly compare with studies of other cancers, which may suggest further research into these observations is warranted.

More research has evaluated miR-155 and the role in oral cancer disease progression [23-25]. These findings have revealed miR-155 expression is correlated with poor clinical prognosis and disease progression [26,27]. The results of this study demonstrated that the most rapidly growing cell lines (CAL-27, SCC-4) exhibited the highest RFE of miR-155, which may suggest these findings support previous observations in other oral tumors.

Finally, the exact role of miR-365 in head and neck cancers remains unknown. Research suggests miR-365 may inhibits growth, invasion, and metastasis of some cancers, such as lung cancer [28]. However, other studies have suggested miR-365 may be associated with poor outcomes and survival in pancreatic cancers [29]. The results of this study demonstrated that only the rapidly dividing cell lines CAL-27 and SCC-4 had moderate or high expression of miR-365, while the relatively slower growing cell lines all exhibited low expression. This may suggest that miR-365 functions in oral cancers in a similar manner to pancreatic cancers – although more research into these observations is clearly warranted.

5. Conclusions

To date, only one study had demonstrated the secretion of oncosomes from cultured OSCC cells [30]. This study demonstrated that exosomes can be successfully isolated from OSCC conditioned media and that microRNA expression can be quantified and evaluated using TaqMan microRNA assays. While more investigation will be needed, potential correlations

between miRNA levels and proliferation rates have been observed that suggest miR-21, miR-155 and miR-365 may be useful biomarkers to evaluate potential growth and proliferation of oral cancers.

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