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Research Article

Differential expression of miR-21, miR-133 and miR-155 from exosome fractions isolated from oral squamous cell carcinomas in vitro

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Exosomes derived from oral cancer cells are membranous vesicles secreted into the surrounding extracellular environment, which are now known to regulate and modulate oral squamous cell carcinoma (OSCC) progression through the horizontal transfer of bioactive molecules, including proteins, lipids and microRNA (miRNA). To date, only one study has demonstrated the secretion of exosomes from cultured OSCC cells, which could potentially facilitate research and possible new treatment modalities. Based upon this information, the primary goal of this study was to examine the potential to isolate and evaluate exosomes from oral cancer cell lines, as well as normal non-cancerous controls. The OSCC cell lines SCC4, SCC9, SCC15, SCC25, CAL27 and the normal gingival cell line HGF-1 were cultured for supernatant collection, which was subsequently centrifuged to remove all intact, but non-adherent cells. RNA was then extracted from the supernatant, as well as from the cytoplasm from each cell line. Molecular screening using primers specific for miRNA to miR-16, -21, -122, -133 and -155 revealed differential expression of miR-21, miR-133 and miR-155 in the cellular fraction of the OSCC cell line, with differential expression of miR-16 in HGF-1 cells. Analysis of supernatant fractions required repeated concentration via centrifugation to detect exosome miRNA, including miR-21, miR-133 and miR-155 from SCC25 supernatant but only miR-16 was detected in the supernatant from HGF-1 cells. Because most cases of OSCC are detected in advanced stages, finding a reliable, non-invasive early stage diagnostic marker would facilitate screening and increase possible treatments.

Keywords: exosomes, miR-21, miR-133, miR-155, microRNA, HGF, oral cancer

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Introduction

Oral cancer is the sixth most common cancer in the United States, with oral squamous cell carcinoma (OSCC) in ~90% of oral cancer patients [1,2]. If OSCC is detected at the early stages, the 5-year survival rate is close to 80% [3,4]. However, if OSCC is detected at the later stages, the 5-year survival rate decreases to 20–40% [5,6]. This supports the necessity for early detection methods for increasing long-term patient survival. The detection of microRNAs (miRNAs) in human saliva has recently become an emerging field for monitoring oral diseases using salivary diagnostics [7,8]. Exosomes are 40-120 nm membranous vesicles that contain unique subsets of miRNA [9]. Most cell types secrete exosomes and they can be found in abundance in body fluids such as blood, urine, and saliva [10]. Exosomes derived from oral cancer cells, also called Oncosomes, secreted into the surrounding extracellular environment, are now known to regulate and modulate OSCC progression through the horizontal transfer of bioactive molecules, including proteins, lipids and miRNA [11,12]. Studies that are able to demonstrate the secretion of exosomes from cultured OSCC cells could potentially facilitate research and possible new treatment modalities.

Studies have recently demonstrated that discriminatory salivary biomarkers can be readily detected upon the development of systemic diseases like pancreatic cancer, breast cancer, lung cancer, and ovarian cancer [13-16]. The challenge is learning more about how discriminatory biomarkers for diseases developing distally from the oral cavity would appear in saliva [17]. Exosomes are likely biomarker candidates as more research is finding out that functional miRNA can be isolated from saliva samples [18]. Very little is known, however, about which miRNAs are up-regulated or down-regulated in oral cancer and whether or not salivary biomarkers can be used as an oral cancer diagnostic tool [19].

Based upon this information, the primary goal of this study was to examine the potential to isolate and evaluate exosomes from oral cancer cell lines, as well as normal non-cancerous control and explore the potential for salivary exosomes to be used in the future as a reliable, non-invasive early stage diagnostic marker that would facilitate screening and increase possible OSCC treatments.

Materials and Methods

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Cell culture and cell lines

The human oral squamous cell carcinoma cell lines SCC4 (CRL-1624), SCC9 (CRL-1629), SCC15 (CRL-1623), CAL 27 (CRL-2095) and SCC25 (CRL-1628), were obtained from American Type Culture Collection (ATCC: Manassas, VA). CAL 27 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.0 mM L-Glutamine adjusted to contain 3.7 g/L sodium bicarbonate, 4.5 g/L glucose, and 110 mg/L sodium pyruvate, obtained from HyClone (Logan, UT). SCCC4, SCC9, SCCC15 and SCC25 cells were maintained in a 1:1 mixture of DMEM and Ham's F12 medium with 2.5 mM L-Glutamine, modified to contain 15 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate.

The normal oral gingival fibroblast cell line, HGF-1 (CRL-2014), was also obtained from American Type Culture Collection (ATCC: Manassas, VA). HGF-1 cells were maintained in DMEM with 4 mM L-Glutamine, adjusted to contain 3.7 g/L sodium bicarbonate and 4.5 g/L glucose, from HyClone (Logan, UT). All cell culture media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 g/mL) solution and 10% fetal bovine serum (FBS) obtained from HyClone (Logan, UT). Cells were cultured in 75 cm^2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO2 in humidified chambers.

RNA isolation

To evaluate microRNA, cellular and supernatant (exosome-containing) fractions were isolated using centrifugation 2.1 RCF or g (4,800 RPM × 10 minutes). RNA was isolated from 1.5×10^7 cells from all cell lines (SCC4, SCC9, SCC15, SCC25, CAL27 and HGF-1) after 72 hours of cell growth using ABgene Total RNA Isolation Reagent (Epsom, Surrey,UK) and the procedure recommended by the manufacturer. RNA concentration and purity were calculated using UV spectroscopy. The absorbance of diluted RNA samples (10 μL of RNA sample in 490 μL nuclease-free water, pH 7.0) was measured at 260 and 280 nm. RNA purity was determined by calculating the ratio of A260:A280, which should be > 1.80. Concentration for RNA samples was determined by the A260 reading of 1 = 40 μg/mL RNA, based on an extinction coefficient calculated for RNA in nuclease-free water. Concentration was calculated as 40 × A260 absorbance measure x dilution factor (50). Total yield was determined by concentration × sample volume in mL. (Example: RNA standard A260 = 0.75; Concentration = 40 × 0.75 × 50 = 1,500 μg/mL; Yield = 1,500 μg/mL * 1.0 mL = 1,500 μg or 1.5 mg RNA). Similar RNA concentrations were obtained from each cell line, which ranged from 876 – 955 ng/L. Analysis of A260/A280 ratio,

confirmed the purity, which ranged between 1.66 and 1.88.

The exosome-containing supernatant fractions were prepared as described above (centrifugation at 2,100 g (RCF) × 10 minutes and were then serially centrifuged at 4,800 g (RCF) * 10 minutes and then 10,000 g (RCF) × 15 minutes to ensure no cells or cellular fractions were present. The identical reagent amounts and procedures were then used to isolate RNA from the processed exosome-containing supernatant fractions, although this did not include counting cells since these had been removed through centrifugation prior to this procedure. RNA concentrations obtained from the supernatant fractions were significantly lower, ranging between 88 – 191 ng/L. Purity ranged between 1.47 – 1.72, which was within the acceptable range for the one-step RT-PCR kit utilized (recommended range: 1.5 – 2.0)

Quantitative Real time PCR (RT-PCR)

Reverse transcription and polymerase chain reaction (RT-PCR) was performed with the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) on both the cellular and exosome-containing supernatant fractions. The primers for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [20] were used to confirm cellular RNA isolation as well as to confirm the absence of cells from the exosome-containing supernatant fractions, which were synthesized by SeqWright (Houston, TX) (Table 1).

In brief, one μg of template (total) RNA was used for each reaction from the cellular RNA extractions derived from each cell line. The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C. Thirty five amplification cycles were run, consisting of 20 second denaturation at 94°C, 30 seconds of annealing at 58.2°C, and 6.5 minutes of extension at 72°C. Final extension was run for 5 minutes at 72°C. Reaction products were separated by gel electrophoresis using Reliant 4% NuSieve R3:1 Plus Agarose gels (Lonza: Rockland, ME). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY). Quantifictation of RT-PCR band densitometry was performed using Adobe (San Jose, CA) Photoshop imaging software, Image Analysis tools.

The sequences of primers used for the experimental screening for microRNA specific to miR-16 [21], miR-21 [22], miR-133 [23], and miR-155 [24] were listed in table 1.

Name	Sequence	Tm(℃)	Optimal Tm(℃)
GAPDH-Forward	ATCTTCCAGGAGCGAGATCC	58.2	53.2
GAPDH-Reverse	ACCACTGACACGTTGGCAGT	60.6	
miR-16-Forward	TAGCAGCACGTAAATATTGGCG	60.8	54.3
miR-16-Reverse	TGCGTGTCGTGGAGTC	59.3	
miR-21-Forward	GCCACCACACCAGCTAATTT	60.4	55.5
miR-21-Reverse	CTGAAGTCGCCATGCAGATA	60.4	
miR-133-Forward	CCGGTTAACTCGAGCTCTGTGAGAGGTTAGTCAG	71.9	65.7
miR-133-Reverse	CTAGCTAGGAATTCTGTGACCTGTGAACTTGGGC	70.7	
miR-155-Forward	TTAATGCTAATTGTGATAGGGGT	57.4	50.6
miR-155-Reverse	CCTATCACAATTAGCATTAATT	55.6	

Table 1. Sequences of quantitative Real time PCR primers

Results

These results demonstrated that RNA concentrations from the cellular fractions were significantly higher, ranging from 876 – 955 ng/uL (Ave.=918.2 ng/uL), compared with those from the exosome-containing supernatant fractions, which ranged between 88 – 191 ng/uL (Ave.= 129.1 ng/uL). These data suggested that standard RT-PCR may not be sensitive enough to detect RNA at concentrations approximately seven-fold lower than recommended (1 ug/uL). To test this hypothesis, PCR signals were evaluated after serially combining fractions each derived from 1 mL aliquots of centrifuged supernatant (Figure 1). These results demonstrated that RT-PCR performed on RNA isolated from 1.0 to 3.5 mL of supernatant remained below the limit of detection (bLOD), but serial combination of 4.0 – 5.0 mL of centrifuged supernatant was sufficient to produce an RT-PCR signal from the normal HGF-1 cell line using miR-16 primers.

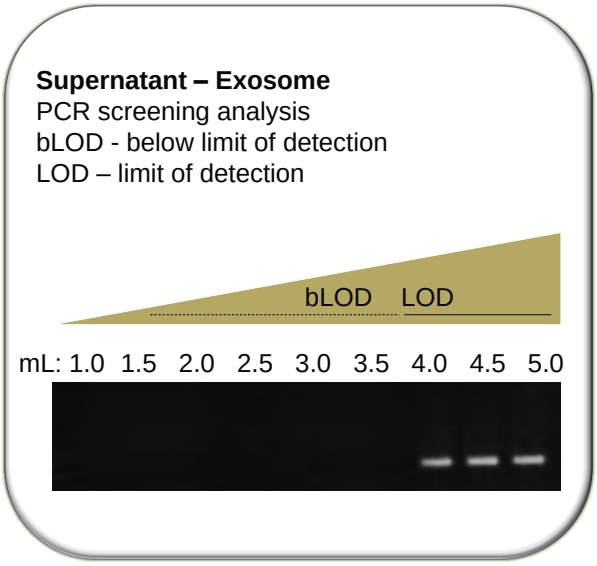

Figure 1. Screening of HGF-1 (normal cell) supernatant for miR-16. RNA concentrations from the non-cellular, exosome-containing supernatant fractions averaging 129.1 (range 88 – 191 ng/uL) were significantly lower than cellular RNA concentrations, averaging 918.2 ng/uL (range 876-955). The low levels of RNA in the non-cellular, exosome-containing supernatant fraction (below the limit of detection, bLOD) required the serial combination of multiple supernatant 1mL fractions for sufficient concentrations of microRNA to enable PCR detection of miR-16 from normal gingiva HGF-1 cells.

Once these parameters were established, molecular screening using primers specific for miRNA to miR-16, miR-21, miR-133 and miR-155 were performed on all cell lines, including the oral squamous cell carcinoma cell lines SCC4, SCC9, SCC15, SCC25, CAL27 and the normal gingival cell line HGF-1 (Figure 2). These results demonstrate expression and extracellular export of miR-16 from the supernatant of HGF-1 only. Differential expression of miR-21, miR-133 and miR-155 was observed in both the cellular and supernatant fractions of the oral cancer cell lines and was not observed in the HGF-1 (normal cell) control. More specifically, miR-21 was expressed and exported into the supernatant from most of the oral cancer cell lines, including SCC4, SCC9, SCC25 and CAL27 – although it was not observed with SCC15. Positive results using miR-133 were also observed from the supernatant derived from SCC4, SCC9, SCC25 and SCC15 – although it was not observed with CAL27. Finally, PCR using miR-155 revealed expression and secretion among SCC4, SCC9 and SCC25 cells – but not in either CAL27 or SCC15.

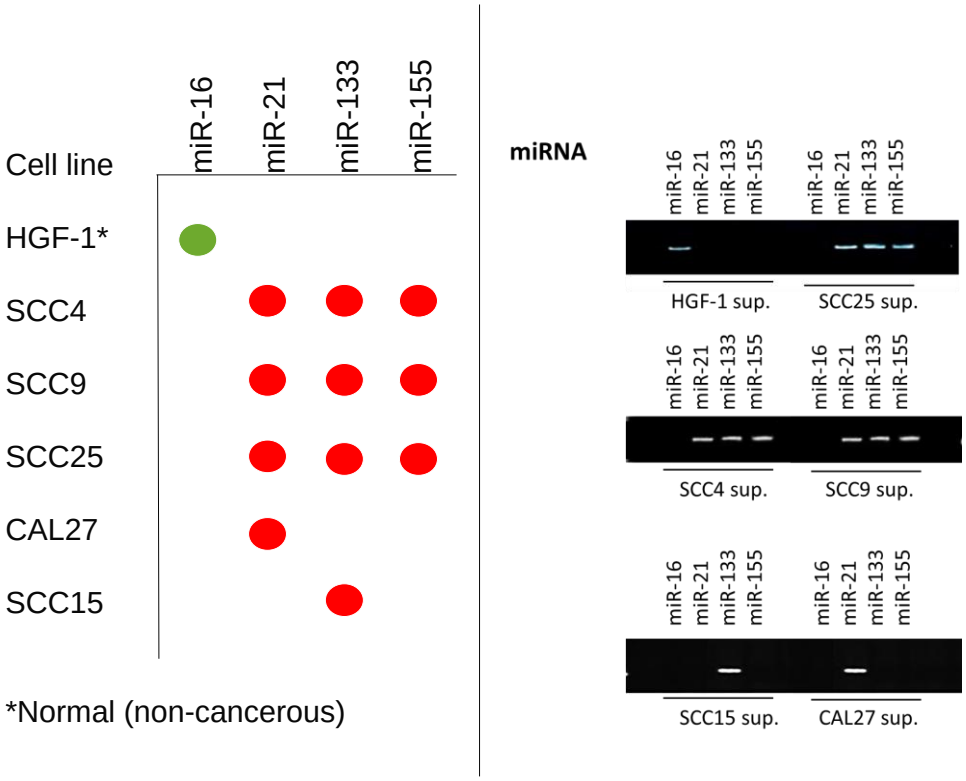

Figure 2. Screening of supernatant from all oral cancer cell lines for microRNA. Using the established parameters (LOD>4mL) centrifuged and serially concentrated RNA from supernatant fractions was screened using RT-PCR for presence of expressed and exported miRNA-16,-21,-133, and -155. miR-16 was detected only from supernatant derived from HGF-1 (normal) cells, while differential results were obtained from SCC4, SCC9, SCC15, SCC25 and CAL27 cells. Positive expression of miR-21 and miR-133 was found among most oral cancer cells (four out of five) with more limited miR-155 expression (three out of five).

Discussion

Based upon the paucity of evidence regarding which miRNAs are up-regulated or down-regulated in oral cancer and whether or not salivary biomarkers can be used as an oral cancer diagnostic tool, the primary objective of this study was to examine the potential to isolate and evaluate exosomes from oral cancer cell lines, as well as normal non-cancerous cell controls. This information could be useful to determine if salivary exosomes might be used in the future as a reliable, non-invasive early stage diagnostic marker that would facilitate screening and increase early diagnosis and thereby allow more effective treatments. The results of this study indicated

that exosomes and microRNA from oral cancer cells in culture can, in fact, be isolated and screened using ordinary laboratory equipment and procedures. Furthermore, these results correspond with new evidence and pilot studies that have suggested saliva-derived miRNA and exosomes may be useful as biomarkers for oral cancers [25,26].

This study also revealed that microRNA expression in oral tumors may be variable and differential, even among commercially available oral cancer cell lines, which suggest that further research is needed to determine which miRNA correspond with potential clinical diagnostics and therapeutic treatments [27,28]. For example, the results of this study appear to confirm the expression of miR-21, miR-133 and miR-155 in most of the oral cancers screened – a result that corresponds with other recent studies of oral cancer [22,29-31]. However, additional studies are now revealing ever more complex and nuanced miRNA expression patterns that may reveal other potential biomarkers for oral cancer, including miR-139, miR-223, and miR-375 [32-36].

Conclusion

This study showed that miRNA from exosomes can be isolated and detected from cultured oral cancer supernatant; however, this process requires repeated concentration to enable detection. The detection of miR-16 only in normal non-cancerous cell supernatant (exosomes) appears to confirm a previous report of five consistent, abundantly expressed microRNAs from human whole saliva (miR-16, miR-24, miR-191, miR-203, miR-223) taken from healthy patients [37]. Although these data provide some evidence for the use of miR-21, -133, and -155 as oral cancer biomarkers, these findings raise more questions as to why these were differentially expressed in oral squamous cell carcinoma supernatant (exosomes). This may suggest more information is needed to understand why these particular miRNAs are differentially regulated in oral cancer cells lines. These data contribute important data towards the elucidation of reliable, non-invasive early stage diagnostic markers that may facilitate screening non-invasive saliva-based screening. This study supports the initial finding that tumor-derived exosomes can be analyzed from in vitro cell cultures, which may allow for further development of discriminatory biomarkers from other pre-malignant and malignant cell cultures that can be applied to saliva and other fluid diagnostic platforms.

Competing interests

The authors declare that they have no competing interests.

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