Differential exosome miRNA expression in oral cancer stem cells

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Abstract

**Background:** Oral squamous cell carcinomas (OSCC) secrete exosomes into the surrounding extracellular environment to promote the horizontal transfer of bioactive molecules including microRNA (miRNA). The primary objective of this study was to explore potential differences in miRNA content between OSCC and OSCC stem cells.

**Methods:** The OSCC cell lines SCC4, SCC15, SCC25 and CAL27 were used in these studies. The corresponding OSCC stem cells that demonstrated phenotypic adhesion independent tumor spheres (AiTS) were manually isolated. All cells were cultured in DMEM containing 10% exosome-free fetal bovine serum. Exosomes were isolated using Total Exosome Isolation reagent (Invitrogen) and RNA was purified using Total Exosome RNA isolation kit (Invitrogen). Exosome miRNA content was evaluated using miRNA Advanced Taqman Assays for miR-21, −155, −133, −34, −31, −32, and −365. The fold change of miRNA content was calculated using the comparative CT (ΔΔCT) method using miR-16 as an endogenous control.

**Results:** After successful cell cultures were established, AiTS (cancer stem cells) were manually separated and confirmed using CD133 and Sox-2 biomarkers. Exosomes and extracellular vesicles were successfully isolated from all cell lines and AiTS isolates for miRNA screening. All isolates exhibited miR-16 expression (positive control), but none contained mir-31, −32, or 133a. Differential expression of miR-21, miR-34 and miR-155 were observed with patterns observed among the cancer cell lines which were distinct from the corresponding AiTS isolates.

**Conclusions:** Exosomes isolated from these different OSCC stem cell populations displayed nearly consistent downregulation/loss of miR-21 and miR-34 suggesting the possibility of a unique miRNA profile characteristic of oral cancer stem cells. These findings highlight the need to investigate the comprehensive functions of miR-21 and miR-34 in tumor progression and continued research to refine a miRNA profile that could aid in distinguishing tumors with poor prognosis.

**Keywords:** Cancer stem cells, Oral cancer, Exosomes, miRNA
among early- versus late-stage oral cancers, which may provide the basis for this type of differential screening [15, 16]. Recent efforts from this group have also contributed to the potential pool of relevant microRNA candidates [17, 18]. More specifically, each of these referenced studies has confirmed the expression of miR-21 and miR-155 in the most aggressive and rapidly dividing oral cancer samples — although none have specifically screened for the expression of these microRNAs among cancer stem cell isolates. In addition, at least two of these studies have found significant correlations with miR-34 and oral cancer prognosis or tumorigenesis — although no studies have screened for the expression of this microRNA among cancer stem cell isolates [15, 16]. Based upon these initial observations, the primary objective of this study was to explore potential differences in specific miRNA content and expression (including miR-21, miR-34, and miR-155) between oral cancers and oral cancer stem cells.

**Methods**

**Cell culture**

Three well-characterized oral cancer cell lines SCC4 (CRL-1624), SCC15 (CRL-1623), SCC25 (CRL-1628) and CAL27 (CRL-2095) were obtained from American Tissue Culture Collection (ATCC). SCC4 and SCC15 cells were cultured according to the manufacturer protocol using Dulbecco's Modified Eagle's Medium (DMEM):F-12, supplemented with 400 ng/mL hydrocortisone, 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. CAL27 and SCC25 cells were maintained in DMEM with 10% FBS and 1% Penicillin-Streptomycin. All cells were maintained in tissue culture treated flasks (25 cm²) and dishes (6-well) in humidified chambers at 5% CO₂.

**Isolation of CSC**

CSC were isolated from each of the three oral SCC cell lines grown in culture. Each adhesion dependent cell (ADC) line grew into uniform monolayers, revealing small sub-populations of CSC in adhesion-independent tumor spheres (AiDC-TS) developing in cell clusters that were not contact or adhesion-dependent within the monolayer [19]. Separation of these CSC-AiDC-TS was facilitated using a cell scraper to dislodge these clusters and a micropipette to facilitate transfer the AiDC-TS into new cell culture flasks for experimentation [20].

**Total cellular RNA isolation**

To determine the presence of CSC-specific mRNA biomarkers, RNA was isolated from both ADC and CSC-AiDC-TS using the ABgene Total RNA isolation Reagent kit and the protocol recommended by the manufacturer [18, 20]. Analysis of RNA was accomplished using absorbance readings at 260 nm and 280 nm. RNA quality was measured using the A260:A280 ratio, which should be greater than 1.70. RNA quantity was measured using A260 absorbance reading of 1 = 40 μg/mL RNA, based on the extinction coefficient of RNA in nuclease-free distilled water. Concentrations were determined as 40 x A260 reading multiplied times the dilution factor.

**Reverse transcription polymerase chain reaction (RT-PCR)**

Expression of CSC-specific mRNA biomarkers was determined using RT-PCR on one ug of total RNA using the ABgene Reverse-iT One-Step RT-PCR kit and a Mastercycler gradient thermocycler using the following primers synthesized by SeqWright:

**Control biomarker**

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

GAPDH forward primer: 5′-ATCTTTCCAGGAGCGAGATCC-3′; 20 nt, 55% GC, 66 °C
GAPDH reverse primer: 5′-ACCACTGACACGTTGGCAGT-3′; 20 nt, 55% GC, 70 °C

**Tumor external (surface) biomarker**

CD44 forward primer: 5′-GAAAGGCATCTTATGGATGTGC-3′; 22 nt, 45% GC, Tm: 64 °C
CD44 reverse primer: 5′-CTGTAGTGAAACACAACCC-3′; 20 nt; 45% GC, Tm: 61 °C

**CSC external (surface) biomarker**

CD133 forward primer: 5′-CTCATGCTTGAGAGATCAGGC-3′; 21 nt, 52% GC, Tm: 65 °C
CD133 reverse primer: 5′-CGTTGAGGAAGATGTGCACCAGT-3′; 25 nt, 56% GC, Tm: 72 °C

**Tumor internal biomarker**

c-myc forward primer: 5′-TCCAGCTTTGTACCTGAGAC-3′; 25 nt, 52%GC, Tm 72°C
c-myc reverse primer: 5′-GCTCCAGCAGAAAGGTGATCC-3′; 25 nt, 56%GC, Tm 72°C

**CSC internal biomarker**

Sox2 forward primer: 5′-ATGGGCTCTGTGGTCAAGTC-3′; 20 nt, 55% GC, Tm: 72 °C
Sox2 reverse primer: 5′-CCCTCCCAATTCCTTGAAGAT-3′; 20 nt, 50% GC, 64 °C

**Exosome isolation**

Each cell line (ADC and AiDC-TS) was then cultured in media supplemented with exosome-depleted FBS for 24 h prior to exosome isolation. The supernatant (conditioned media) was removed from each tissue culture
flask and centrifuged at 2000 x g for 30 min to remove any cells or cellular debris. The cell-free supernatant was then added to Total Exosome Isolation reagent from Life Technology and incubated overnight at 4 °C, as directed by the manufacturer protocol. Exosomes and extracellular vesicles were subsequently isolated using centrifugation at 10,000 x g for 60 min at 4 °C. The exosome-containing pellets were then resuspended in 100 uL of 1X Phosphate-Buffered Saline (PBS).

**Exosome RNA isolation**

The exosome resuspension was mixed with an equal volume of 2X Denaturating Solution from Life Technology and incubated on ice for 5 min. An equal volume of Phenol:Chloroform was added prior to centrifugation at 10,000 x g for 15 s. The aqueous (upper) phase was removed, mixed 1:1.25 with 100% ethanol (EtOH). This solution was transferred into a filter for subsequent centrifugation at 10,000 x g for 15 s. Each sample-filter was washed with miRNA Wash Solution 1 from life Technology and centrifuged at 10,000 x g for 15 s. This process was repeated with Wash Solution 2/3. Each sample-filter was subsequently placed into a sterile collection tube with 100 uL of heated RNase water and centrifuged for 30 s to collect the exosomal RNA.

**Exosome analysis**

Exosome isolation was verified using Particle Metrix Nanoparticle Tracking Analysis (NTA) Zeta View software using the manufacturer default setting for extracellular vesicles (EVs) and Nanospheres. Each sample was diluted in sterile 1X PBS to a final concentration of 3.2 x 107 particles/mL to achieve an Average Counted Particles per Frame (ACPF) of 89, well within the recommended particle per frame value range of 40–200. Each measurement involved two cycles scanned at 11 positions with the following settings: Focus – Autofocus; Scattering Intensity – Detected Automatically; Cell temperature – 27.89°C sensed. Following data capture, analysis was performed using ZetaView Software 8.05.10 using the following analysis parameters: Max Area: 1000; Min Area: 5; Minimum brightness: 25; Camera 0.712 μm/px.

**TaqMan microRNA assays**

Analysis of exosomal RNA was accomplished using TaqMan microRNA assays, consisting of Reverse Transcription using 10X Reverse Transcription Buffer, 100 mM deoxyribonucleotide triphosphate (dNTP), RNase inhibitor, and MultiScribe Reverse Transcriptase containing 3 uL of miR specific primer. Settings for the thermocycler were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles that consisted of 15 s at 95 °C and 60 s at 60 °C. A standard curve was derived from a five-fold serial dilution of cDNA from miR-16, which is used as a positive control for exosomal miRNA and the endogenous reference gene [15, 18].

**Statistical analysis**

Any differences in miRNA expression were evaluated using two-tailed t-tests isolated from each cell line (ADC and AiDC-TS). Statistical significance level was set at α = 0.05.

**Results**

Four oral squamous cell carcinoma cell lines (SCC4, SCC15, SCC25 and CAL27) were obtained and cultured to isolate potential CSC from adhesion-independent tumor spheres (Fig. 1). More specifically, as cultures were maintained over time, adhesion-independent tumor spheres (AiTS) developed, which were manually isolated and transferred to separate cell culture flasks for SCC4 (Fig. 1a), SCC15 (Fig. 1b), SCC25 (Fig. 1c) and CAL27 (Fig. 1d) cells. Adhesion dependent (original) and AiTS cultures were then separately cultured and maintained.

In order to determine any differences in mRNA expression and cellular phenotype, RNA was isolated from each of the cell lines and the corresponding AiTS separated from those cell lines (Table 1). These data revealed RNA isolated from each of the original cell cultures had higher concentrations than RNA isolated from the corresponding AiTS for all cell lines. The average RNA concentration from the SCC cell lines was 562 ng/μL (ranging between 278 and 965 ng/μL), which was higher than observed from the AiTS isolated from the corresponding SCC cell lines at 499 ng/μL (ranging between 215 and 892 ng/μL). RNA purity was measured using the ratio of absorbance readings at 260 nm and 280 nm, which revealed an average of 1.68 and 1.53 among the SCC and AiTS isolates, respectively.

To determine if differences in mRNA expression were evident among the SCC and AiTS isolates, RT-PCR was performed on surface biomarkers to distinguish cancer and cancer stem cells (Fig. 2). These data revealed that the SCC cells (SCC4, SCC15, SCC25 and CAL27) expressed the CD44 biomarker, which may be used to distinguish between normal tissue and oral cancers. This expression was also observed among the AiTS from each cell line, although mRNA expression levels appeared to be slightly lower. However, the expression of CD133, which may be used to distinguish between cancer and cancer stem cells was highly expressed among the AiTS,
with much lower (or nearly absent) expression observed among the corresponding cell lines.

To determine if the differential expression of cell surface biomarkers was also correlated with internal biomarkers, the RNA was also screened for c-myc (cancer) and Sox-2 (cancer stem cell) mRNA expression (Fig. 3). These data revealed similar c-myc mRNA expression among both the SCC and AiTS isolates. However, Sox-2 expression was significantly higher among each of the AiTS isolates than was observed among the SCC cell lines.

Each SCC cell line and corresponding AiTS isolate was transferred into exosome-free media for subsequent exosome and RNA isolation (Table 2). In brief, following the exosome extraction protocol, RNA was isolated from each of the corresponding exosome preparations, which revealed similar levels of RNA. The average RNA concentration from the SCC exosome preparation (30.8 ng) was not significantly different from the AiTS exosome preparation (30.7 ng), \( p = 0.9588 \).

To analyze any potential differences in microRNA expression, TaqMan assays for miR-16, miR-21, miR-34 and miR-155 were used to screen the RNA extracted from the exosomal preparations (Fig. 4). In brief, miR-16 was used to normalize the exosomal microRNA expression, which revealed nearly equivalent amounts of miR-21, miR-34 and miR-155 among the SCC exosomal preparations. However, differential expression was observed among the AiTS. For example, miR-21 expression among the exosomal preparations from the AiTS was down-regulated or absent entirely. Similarly, miR-34 expression was also down-regulated or missing among three of the four AiTS (SCC4, SCC15, and SCC25) with a marked up-regulation observed among the SCC25 AiTS. In addition, miR-155 expression was significantly up-regulated among three of the four AiTS (SCC4, SCC15, and SCC25) with the only down-regulation observed among the CAL27 cells.

To confirm the identification of isolated exosomes, Particle Metrix ZetaView Nanoparticle Tracking Analysis (NTA) was used (Fig. 5). In brief, analysis of exosomal preparations diluted to \( 3.2 \times 10^7 \) particles/mL were screened in two cycles and 11 positions to provide verification that the exosomal isolation yielded extracellular vesicles (EVs) and nanoparticles ranging in size between 50 and 200 nm. More specifically, the mean size of EVs from the NTA analysis was 15.9.3 nm +/− 0.95 (STD).

### Table 1 Isolation of RNA from SCC lines and AiTS

| Isolate | RNA concentration | RNA purity (A260/A280) |
|---------|-------------------|-------------------------|
| SCC-4   | 278 ng/μL         | 1.55                    |
| SCC-4 AiTS | 215 ng/μL     | 1.22                    |
| SCC-15  | 317 ng/μL         | 1.63                    |
| SCC-15 AiTS | 251 ng/μL     | 1.42                    |
| SCC-25  | 688 ng/μL         | 1.80                    |
| SCC-25 AiTS | 637 ng/μL     | 1.65                    |
| CAL-27  | 965 ng/μL         | 1.75                    |
| CAL-27 AiTS | 892 ng/μL     | 1.81                    |
| SCC average | 562 ng/μL     | 1.68                    |
| SCC range  | 278–965 ng/μL    | 1.55–1.80               |
| SCC AiTS average | 499 ng/μL     | 1.53                    |
| SCC AiTS range | 215–892 ng/μL  | 1.22–1.81               |

Fig. 1 Isolation of Adhesion-Independent Tumor Spheres (AiTS) from cell cultures. Cell cultures maintained over time developed distinct areas of AiTS, which were subsequently isolated and cultured separately for SCC4 (A), SCC15 (B), SCC25 (C), and CAL27 (D).
with an average volume of 431.6 nm$^3$ $\pm$ 244.9 (STD), which corresponds with known size distributions and parameters for exosomes and extracellular vesicles.

**Discussion**
The main goal of this study was to examine any potential differences in miRNA content between oral cancers and oral cancer stem cells. The preliminary results of this investigation provide initial evidence that some significant and fairly consistent changes in miRNA expression may be found between oral cancers and their respective stem cell sub-populations, which have been found in other cancers and their corresponding cancer stem cells [21, 22]. This may be consistent with the known functions of microRNAs as regulators of several key tumor-related functions, including angiogenesis [23, 24].
Interestingly, these data may provide evidence that miR-21 might be down-regulated among oral cancer stem cell sub-populations, a key regulator of PI3K/Akt pathway [25, 26]. These observations may be consistent with other studies that suggest control and regulation of the PI3K/STAT3/NF-kB signaling pathway may be more important to tumor functions than critical stem cell or cancer stem cell functions [27, 28]. However, this may be among the first studies to confirm this differential expression between sub-populations derived from the same commercially available oral cancer cell lines.

Another novel finding from this study is the observation that miR-34 may also be down-regulated among the oral cancer stem cell sub-populations. Previous studies have demonstrated that miR-34 may be linked with the regulation of the critical tumor suppressor p53 [29, 30]. As both p53 and miR-34 appear to modulate Wnt signaling, which may also regulate proliferation in both cancers and cancer stem cells – this finding may be consistent with the highly proliferative nature of cancer stem cells [31, 32].

One anomalous finding in this study was miR-34 up-regulation among the SCC25 AiTS, with down-regulation observed among the other three SCC AiTS. More detailed genetic analysis has revealed the presence of underlying dissimilar genetic mutations among these cell lines that may have the potential to influence these experimental outcomes. More specifically, SCC25 cells are known to harbor a homozygous deletion p53 not found in SCC4, SCC15 or CAL27 cells that might influence these

| Table 2 | Isolation of RNA from Exosome Preparations derived from SCC lines and AiTS |
|-----------------|-----------------|-----------------|-----------------|
| RNA concentration | Statistical analysis | RNA concentration | Statistical analysis |
| SCC-4 | 29.7 ng | Two-tailed t-test | SCC-4 AiTS | 27.4 ng |
| SCC-4 AiTS | 27.4 ng |  | SCC-15 | 34.1 ng |
| SCC-15 | 35.2 ng |  | SCC-15 AiTS | 31.1 ng |
| SCC-25 | 32.6 ng |  | SCC-25 AiTS | 28.2 ng |
| CAL-27 | 27.4 ng |  | CAL-27 AiTS | 28.2 ng |
| SCC average | 30.8 ng |  | SCC AiTS average | 30.7 ng |
| SCC AiTS average | 28.2–34.1 ng |  | p = 0.9588 |

Fig. 4 TaqMan microRNA assays for exosomal RNA analysis. A. RNA extracted from SCC and AiTS exosomal preparations was screened using TaqMan assays for miR-16, miR-21, miR-34 and miR-155. Expression was normalized to miR-16 (positive control), which revealed similar expression of miR-21, miR-34 and miR-155 among the SCC exosomal preparations. Down-regulation was observed among the AiTS for miR-21 and miR-34 (except SCC25 AiTS). Up-regulation of miR-155 was observed among three of the four AiTS (except CAL27). B. Heat map of microRNA exosomal expression demonstrated differential expression among all AiTS (cancer stem cell) isolates for miR-21, miR-34 and miR-155.
findings through disruption of the positive feedback loop between miR-34 and p53 transcriptional regulation [33, 34].

Among the most interesting findings of this study is the observation of miR-155 up-regulation among the AiTS oral cancer stem cell sub-populations. This may be significant as over-expression of miR-155 appears to directly promote cellular proliferation in oral cancers [35–37]. These observations may conform with additional evidence that has demonstrated miR-155 may be an indicator of poor prognosis among oral cancers [18, 37, 38].

These findings suggest further studies into these phenomena may be warranted and should include primary tumor explants, which may provide more relevant results than these initial findings from commercially available cell lines - that may also be more readily translated into clinical practice. In addition, another major limitation of this study was the financial constraints on the number of microRNA assays that could be evaluated. Future studies may include additional relevant microRNA candidates, which might greatly enhance the prognostic and diagnostic capabilities of oral cancers.

**Conclusions**

This study provides preliminary evidence that oral cancers with sub-populations of cancer stem cells may exhibit differential expression of microRNAs. These data may be valuable not only as a prognostic or diagnostic indicator for oral tumors, but also as part of a larger map of potential pathways that may be active or inhibited in these distinct but overlapping cell populations. More research will be needed to determine the clinical and therapeutic relevance of these findings.

**Abbreviations**

ADC: Adhesion dependent cell; AiTS: Adhesion-independent tumor spheres; ATCC: American Tissue Culture Collection; CSC: Cancer stem cells; dNTP: Deoxyribonucleotide triphosphate; EtOH: Ethanol; FBS: Fetal bovine serum; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; nt: Nucleotide; PBS: Phosphate-buffered saline; RNA: Ribonucleic acid; SCC: Squamous cell carcinoma

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**Authors’ contributions**

MS, TB, DH and BL performed the experimental protocols and data generation. KK and KMH were responsible for project design and supervision. All authors contributed to this manuscript and have read and approved the final manuscript.

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**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
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