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Differential Expression of Cellular and Exosomal MicroRNA Isolated from Oral Cancer Cells and their Resistance to Chemotherapy

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Abstract: MicroRNAs are abundant small non-coding RNA with a variety of known functions, including transcriptional activation and inhibition. Recent evidence has suggested that microRNA expression may influence the responsiveness of some cancers to chemotherapy, including liver and lung cancers. Some evidence has now suggested that specific microRNAs, such as miR-21, miR-155, and miR-375, may influence oral cancer responsiveness to chemotherapy – although much remains to be discovered. Based on the lack of evidence in this area, the primary objective of this study was to evaluate microRNA expression and responsiveness among oral cancers. Five commercially available oral cancer cell lines (SCC4, SCC9, SCC15, SCC25, CAL27) were obtained from ATCC and cultured and chemotherapy resistance to Cisplatin, Fluorouracil or 5-FU and Paclitaxel or Taxol was assessed. Exosomes were then isolated, confirmed, and processed using Particle Metrix Nano Tracking Analysis (NTA) Subsequently, RNA was isolated from both the exosomes and cellular fractions and qPCR screening was performed to determine the expression of microRNA from cellular and exosomal isolates. Growth assays revealed that SCC15 assays were the least resistant, while CAL27 and SCC4 cells exhibited moderate resistance, and SCC9 and SCC25 cells exhibited strong and differential resistance to these chemotherapeutic agents. The screening revealed all cancers expressed miR-21 and miR-133 with differential expressions of miR-27, miR-135, miR-155, and miR-375 observed. However, the most resistant cell lines, SCC9 and SCC25, were the only cells to express miR-375 and were also the only cells that did not express miR-27, suggesting an association between chemotherapeutic resistance and expression of these specific microRNAs. In addition, miR-21 and miR-133 were identified in exosomes from all oral cancers with differential results observed with miR-133 and miR-135-although miR-27 and miR-375 were not found among any exosomes. Although much remains to be elucidated about the functional roles of these differentially expressed microRNAs, the findings of this study suggest that specific microRNAs including miR-27 and miR-375 may, in fact, function in distinct, different, and opposite pathways among these cell lines. Future research endeavors will need to evaluate the potential role of these microRNAs not only to validate their predictive capabilities as biomarkers but also to ascertain which functional pathways may be involved in the development and progression of oral cancers.

Keywords: Oral Cancer, Exosome, MicroRNA

Introduction

MicroRNAs are abundant small non-coding RNA with a variety of known functions, including transcriptional activation and inhibition (Liu *et al*., 2021; Van Meter *et al*., 2020). Research into the functional aspects of microRNA epigenetic regulation has led to discoveries that suggest specific microRNAs, such as miR-21 and miR-155, may be critical mediators of health or disease states including the development, progression, and resolution of chronic inflammation (Mahesh and Biswas, 2019; Dioguardi *et al*., 2020). More recent evidence has suggested that microRNAs may also function in more complex and chronic disease states, including cardiovascular and neurologic diseases, as well as cancer (Wojciechowska *et al*., 2017; Juźwik *et al*., 2019; Verduci *et al*., 2019; Zhang *et al*., 2020).

Recent evidence has suggested that microRNA expression may influence the responsiveness of some cancers to chemotherapy, including liver, lung, and colorectal cancers (Wang *et al*., 2019; Balacescu *et al*., 2018; Iqbal *et al*., 2019). These analyses have led to remarkable discoveries that specific microRNAs may play critical roles in tumor responsiveness to chemotherapies, such as miR-21, miR-155, and miR-375 in lung and other cancers (Gan *et al*., 2017; Shao *et al*., 2019; Bica-Pop *et al*., 2018). Similarly, further studies have confirmed the important roles of miR-21 and miR-155 in chemotherapeutic resistance in other cancers, such as colorectal cancers, while also uncovering novel microRNAs that may also play a role in these phenotypes such as miR-27 (Zhang *et al*., 2019; Jung *et al*., 2020). In addition to miR-21 and miR-155, other microRNAs have been demonstrated as critical mediators of pathogenesis and chemotherapy resistance, although their expression may be restricted to specific types of cancers, such as Let-7d and female-associated malignancies including breast and ovarian cancer (De Santis and Götte, 2021; Gunel *et al*., 2019).

Some evidence has now suggested that specific microRNAs, miR-21, miR-155, and miR-133, may influence oral cancer responsiveness to therapeutic agents-although much remains to be discovered (Hunsaker *et al*., 2019). Several independent studies have found additional evidence that some microRNAs may not be expressed ubiquitously in oral tumors, but rather may be differentially expressed either over time or in distinct tumors based upon phenotype and can be either up or down-regulated (Rishabh *et al*., 2021; Duncan *et al*., 2021). One such microRNA is miR-135, which has been demonstrated to upregulate proliferation and down-regulate apoptosis in some oral cancers while modulating the response of these tumors to Cisplatin (Zhang and Wang, 2018; Wang and Zhang, 2018).

Some new evidence has emerged that resistance to chemotherapy may be specifically determined by "pivotal" microRNAs, which appear to be active in several different types of cancer (Geretto *et al*., 2017). For example, chemotherapeutic resistance to 5-Fluorouracil (5-FU) among various cancers appears to be integrally related to the expression of miR-21, miR-27, and miR-155 (Ghafouri-Fard *et al*., 2021; Lin *et al*., 2020; Wang *et al*., 2021). Some evidence has suggested that resistance to Cisplatin among these same tumors may be associated with differential expression of miR-16 miR-21 and miR-155 (Wang *et al*., 2020; Sayyed *et al*., 2021). Other studies from this group have identified both miR-365 and miR-720, which appear to modulate oral cancer growth and proliferation, but are not responsive to chemotherapy (Coon *et al*., 2020; Coon and Kingsley, 2021; Graves *et al*., 2020).

However, to date, no comprehensive microRNA screening has been done to determine the association between the magnitude of oral cancer responsiveness and the specificity of chemotherapeutic resistance among well-characterized oral cancer cell lines. Based on the lack of evidence in this area, the primary objective of this study was to evaluate the microRNA expression of miR-21, miR-27, miR-133, miR-135, miR-155, and miR-275 with chemotherapeutic responsiveness among oral cancers.

Materials and Methods

Experimental Cell Lines

Oral squamous cell carcinoma cell lines were obtained from American Culture Tissue Collection (ATCC; Manassas, VA), including SCC4 (CRL-1624), and SCC9 (CRL-1629), SCC15 (CRL-1623), SCC25 (CRL-1628) and CAL27 (CRL-2095). Each cell line was cultured according to the manufacturer's recommended protocols, which included Dulbecco's Modified Eagle's Medium (DMEM) for CAL27 cells and DMEM: F12 for SCC4, SCC-9, SCC15, and SCC25 cells with the addition of 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin from Thermo Fisher Scientific (Fair Lawn, NJ). Cells were cultured in tissue culture-treated flasks and kept in a humidified Biosafety Level 2 incubator at 37°C with supplemental CO_2 at 5%. In addition, each cell line was previously screened using the Short Tandem Repeat method to ensure the validity of each cell line and prevent usage of cell lines with cross-contamination (Coon *et al*., 2020; Coon and Kingsley, 2021).

Proliferation Assays

Three-day proliferation assays were performed using each cell line to establish baseline growth rates. In brief, cells were seeded into 96-well plates at a concentration of 1.2×10^5 cells per mL with specific endpoints of 24, 48, and 72 h. Experimental assays to determine potential chemotherapy resistance were subsequently performed using anti-tumor agents Cisplatin (MW 300.5) or cis-diamine-dichloro platin from Selleck Chemical (Houston, TX), Paclitaxel (MW 853.9) from MP Biochemical (Santa

Ana, CA) and a standardized mitotic (selective MEK) inhibitor PD98059 (MW 267.3; 167869-21-8) from Selleck Chemical (Pittsburgh, PA). Three concentrations (Low: 1 ng/mL, Mid: 5, and High: 10 ng/mL) were utilized to mimic physiological dosages and bioavailability studies. Three independent experiments were conducted on each cell line at each concentration of all three inhibitors, which also included a negative control (no treatment).

Cellular RNA Isolation

Cellular RNA was extracted from each cell line for analysis. In brief, cells were processed using the phenol: Chloroform extraction method using TRIzol reagent from Fisher Scientific (Fair Lawn, NJ), which included media removal (supernatant aspiration), lysis with the TRIzol reagent, and transfer to a sterile microcentrifuge tube. To each sample containing 1.0 mL of TRIzol-cellular lysate, 200 uL of molecular biology grade chloroform from Sigma-Aldrich (St. Louis, MO) was added, mixed thoroughly, and incubated for five minutes at room temperature. Samples were then centrifuged at 12,000 × *g* or RCF (relative centrifugal force) for 15 min at 4°C. The RNA-containing upper phase was removed and transferred to a sterile microcentrifuge tube and an equal volume of molecular biology grade isopropanol from Sigma-Aldrich (St. Louis, MO) was added and mixed. Following incubation on ice for five minutes, samples were centrifuged for ten minutes at 12,000 × *g* (RCF for 10 min at 4°C. The supernatant was carefully aspirated and the pellet was washed with 70% molecular biology grade ethanol from Sigma-Aldrich (St. Louis, MO) and centrifuged again for 10 min. The supernatant was carefully aspirated and the RNA-containing pellet was resuspended in 100 uL of nuclease-free, molecular biology grade water and stored at -20°C.

RNA Analysis

The quality and quantity of RNA were determined using a NanoDrop 2000 spectrophotometer from Fisher Scientific (Fair Lawn, NJ). In brief, absorbance readings at A260 nm were measured and RNA concentration was calculated using the standard formula of 1 unit of absorbance at this wavelength corresponding with 40 ugs of RNA per mL. RNA concentrations generally range between 1000 ng and 10,000 ng/mL for samples taken from cellular lysates. Absorbances at A260 can also be used in conjunction with absorbances at A230 and A280 nm, which were also measured to provide estimates of protein contamination. A 260/280 ratios between 1.8 and 2.1 are considered pure and acceptable for molecular biology applications, such as qPCR screening. A 260/230 ratios over 2.0 demonstrate low phenol carryover and contamination.

Cellular cDNA Synthesis and qPCR Screening

Cellular RNA was reverse transcribed into cDNA using the ABgene Reverse-iT One-Step RT-PCR kit from Thermo Fisher Scientific (Fair Lawn, NJ). Briefly, 1.0 ug of cellular RNA was added to 12.5 uL of 2X Reddy Mix (RT-PCR Master mix), 1.0 uL each of sense and antisense primer, 1.0 uL of RTase blend, and nuclease-free water and processed using a Mastercycler gradient thermal cycler from Eppendorf (Hamburg, Germany). The setting included 30 min at 47°C, followed by 2 min at 94°C and 40 cycles of denaturation for 20 sec at 94°C, annealing for 30 sec at the appropriate primer temperature, and extension for 60 sec at 72°C. The quality and quantity of cDNA synthesis were determined using a NanoDrop 2000 spectrophotometer, as described above.

Each cDNA sample was screened for microRNA expression using quantitative Polymerase Chain Reaction (qPCR) and primers specific for each microRNA, synthesized by SeqWright from Fisher Scientific (Fair Lawn, NJ). Each sample was run in duplicate, using two positive controls, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) for the metabolic standard and beta-actin for the cytoskeleton and structural standard. The reaction was performed using the SYBR Green qPCR Master Mix from Fisher Scientific (Fair Lawn, NJ), which was composed of 2X Absolute SYBR green master mix, forward and reverse primers, and cDNA (1.5 ng) and distilled nuclease-free water. Settings included enzymatic activation for 15 min at 95°C and 40 cycles of 15-sec denaturation at 95°C, annealing for 30 sec at the primer pair-specific temperature listed below, and extension for 30 sec at 72°C.

GAPDH
GAPDH forward: 5′-ATC TTC CAG GAG CGA GAT CC-3′; 20 nt, 55% GC, Tm 66°C
GAPDH reverse: 5′-ACC ACT GAC ACG TTG GCA GT-3′; 20 nt, 55% GC, Tm 70°C
Optimal Tm: 61°C

Beta-actin
Beta-actin forward, 5′-GTG GGG TCC TGT GGT GTG-3′; 18 nt, 67% GC, Tm: 69°C
Beta-actin reverse, 5′-GAA GGG GAC AGG CAG TGA-3′, 18 nt, 61% GC, Tm: 67°C
Optimal Tm: 61°C
miR-16 forward: 5’-TAG CAG CAC GTA AAT ATT GGC G-3’; (22 nt) Tm: 60.8°C
miR-16 reverse: 5’-TGC GTG TCG TGG AGT C-3’; (16 nt) Tm: 59.3°C
Optimal Tm (PCR): 54.3°C
miR-21
miR-21 forward: 5'-GCC ACC ACA CCA GCT AAT TT-3'; 20 nt; 50% GC, Tm: 66°C
miR-21 reverse: 5'-CTG AAG TCG CCA TGC AGA TA-3’; 20 nt; 50% GC; Tm: 65°C
Optimal Tm (PCR): 60°C

miR-27
miR-27 forward: 5’-ATA TGA GAA AAG AGC TTC CCT GTG-3’; 24 nt, 42% GC, Tm: 61°C
miR-27 reverse: 5’-CAA GGC CAG AGG AGG TGA G-’3’; 18 nt, 61% GC, Tm: 67°C
Optimal Tm (PCR): 56°C

miR-133
miR-133 forward: 5’-CCG GTT AAC TCG AGC TCT GTG AGA G-3’; 25 nt, 56% GC Tm: 71°C
miR-133 reverse: 5’-CTA GCT AGG AAT TCT GTG ACC TGT G-’3’; 25 nt, 48% GC, Tm: 66°C
Optimal Tm (PCR): 60°C

miR135
miR-135 forward: 5'-CGA TAT GGC TTT TTA TTC CTA -3'; 21 nt, 33% GC, Tm: 56°C
miR-135 reverse: 5’-GAG CAG GGT CCG AGG T -3’; 16 nt, 69% GC, Tm: 67°C
Optimal Tm (PCR): 51°C
miR155
miR-155 forward: 5'-TTA ATG CTA ATT GTG ATA GGG GT-3'; 23 nt, 35% GC, Tm: 61°C
miR-155 reverse: 5’-CCT ATC ACA ATT AGC ATT AAT T-3’; 22 nt, 27% GC, Tm: 55°C
Optimal Tm (PCR): 50°C
miR-375
miR-375 forward: 5'-GGCTCTAGAGGGGACGAAGC-3'; 20 nt, 65% GC, Tm: 70°C
miR-375 reverse: 5’-GGCAAGCTTTTTCCACACCTCAGCCTTG-3’; 28 nt, 54% GC, Tm: 74°C
Optimal Tm (PCR): 65°C

Exosome Isolation and Analysis

To evaluate the exosomal microRNA content, exosomes and extracellular vesicles were isolated from each cell culture. In brief, cell culture media was aspirated and cells were washed with 1X Phosphate Buffered Saline (PBS) from Thermo Fisher Scientific (Fair Lawn, NJ). The appropriate media supplemented with 1% penicillin-streptomycin and 10% exosome-depleted FBS from Gibco (Amarillo, TX), which has been demonstrated to remove >90% of exogenous exosomes and extracellular vesicles. Cells were cultured for a minimum of 24 h before supernatant harvesting and exosome isolation.

After 24 h, the conditioned media was removed from each cell culture and centrifuged at 2,000 × *g* (RCF) for 30 min to remove cellular debris, according to the manufacturer protocol (Coon *et al*., 2020; Coon and Kingsley, 2021). The supernatant was carefully removed and transferred to a new sterile microcentrifuge tube and mixed with 0.5 volumes of Total Exosome Isolation reagent from Invitrogen (Waltham, MA) and incubated overnight at 4°C. Following the overnight incubation, each sample was centrifuged at 10,000 × *g* (RCF) or 60 min at 4°C. The supernatant was carefully aspirated and the exosome-and microvesicle-containing pellet was resuspended in 100 uL of sterile 1X PBS for downstream analysis, protein isolation, and RNA extraction.

Analysis of exosomes and extracellular vesicles was performed using Particle Metrix-Nanoparticle Tracking Analysis from ZetaView (Inning am Ammersee, Germany), and the manufacturer recommended protocol for analysis of Extracellular Vesicles (EV) and nanospheres. Samples were diluted using 1X sterile PBS to an Average of Counted Particles per Frame (ACPF) of approximately 100, which is within the optimal particle concentration range established by the manufacturer between 40-200. This corresponds to roughly 3.0×10^7 particles per mL for each sample. This analysis provides both the peak and mean diameter of EVs, including exosomes and nanospheres.

Exosomal RNA and Protein Purification

Exosome-containing samples were then processed using the Total Exosome RNA and Protein Isolation Kit from Invitrogen (Waltham, MA) according to the manufacturer's recommended protocols. Briefly, RNA was extracted from samples by adding an equal volume of 2X Denaturing Solution, vortexing to mix, followed by incubation on ice for five minutes. An equal volume of Acid-Phenol: Chloroform was added and mixed by vortexing, followed by centrifugation at 10,000 x *g* (RCF) for five minutes. The resulting phase separation allows for the separation of proteins into the lower protein-containing phase and RNA in the upper aqueous phase.

The lower protein-containing phase was processed using the Bradford Protein Assay from BioRad (Hercules, CA). In brief, blank samples were made using 0.5 uL of water and dye reagent, with exosome samples prepared using 0.5 uL of protein sample and 25 uL of Coomassie dye to obtain colorimetric blue (590 nm) shift. Following the confirmation of protein isolation, each sample was processed for Western blot using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using precast 12% gels from BioRad and transferred to a nitrocellulose membrane before incubation with anti-CD63 antibody and visualization with anti-Horseradish Peroxidase (HRP) conjugated secondary antibody.

The upper RNA-containing phase was transferred to a new sterile microcentrifuge tube and mixed with 1.25 volumes of 100% Ethanol. 700 uL of each mixture was transferred to a Filter Cartridge secured in a Collection Tube and centrifuged at 10,000 × *g* (RCF) for 15 sec. The flow-through was discarded and the Filter Cartridge was placed back into the Collection Tube with 500 uL of Wash Solution and centrifuged at 10,000 × *g* (RCF) for 15 sec. Once again the flow-

through was discarded and Wash Solution was added with final centrifugation at 10,000 × *g* (RCF) for 60 sec. The Filter Cartridge was then placed into a fresh sterile Collection Tube with 50 uL of Elution Solution and centrifuged at 10,000 × *g* (RCF) for 30 sec. This step was repeated and the extracted RNA-containing solution was stored at -20°C for further analysis and processing, as described above.

cDNA Synthesis and MicroRNA Amplification

The TaqMan Advanced miRNA cDNA synthesis kit from Applied Biosystems (Waltham, MA) was used to process each sample. In brief, samples were thawed and 2 uL placed into each corresponding reaction plate well with 3 uL of Poly(A) Reaction Mix, which was prepared using 10X Poly(A) buffer, Poly(A) enzyme, ATP and RNase-free water according to the manufacturer protocol. Each plate was centrifuged using a MicroPlate Centrifuge from Thermo Fisher Scientific (Fair Lawn, NJ). Polyadenylation was accomplished using a thermocycler at 37°C for 45 min, followed by a stop reaction at 65°C for ten minutes. To each sample, 10 uL of Ligation Reaction mix (containing 5X DNA ligase buffer, 25X ligase adaptor, RNA ligase, and RNase-free water) was added, vortexed, and then centrifuged before thermocycling at 16°C for 60 min. Finally, 15 uL of Reverse Transcription (RT) Reaction mix (containing 5X RT buffer, 20X Universal RT primer, 10X RT enzyme mix, dNTP, and RNase-free water) was added to each sample, vortexed, and centrifuged before reverse transcription in a thermal cycler at 42°C for 15 min and a stop reaction at 85°C for five minutes.

To further amplify any low-expression microRNA targets, cDNA from the amplification reaction can be used with miR-Amp Reaction Mix (containing 2X miR-Amp Master Mix, 20X Primer Mix, and RNase-free water). In brief, in a new reaction plate, 5 uL of cDNA from the RT reaction was mixed with 45 ul of the miR-Amp Reaction mix, vortexed, and then centrifuged before amplification in a thermal cycler using one cycle of 95°C for five minutes to activate the enzyme, followed by 14 cycles of denaturation at 95°C and annealing/extension at 60°C for 30 sec, followed by a stop reaction at 99°C for ten minutes.

qPCR Screening

TaqMan Advanced Master Mix was prepared according to the manufacturer protocol (containing 2X Master Mix, 20X Advanced miRNA Assay, and RNase-free water) and 15 uL were added to each well of a new 96-well reaction plate with 5 uL of 1:10 diluted cDNA template from each sample. Each plate was vortexed, centrifuged, and then placed into a thermal cycler with enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for one second, followed by annealing and extension at 60°C for 20 sec. TaqMan microRNA assays for miR-21, miR-31, miR-133, and miR-135 were performed.

Statistical Analysis

Differences between absorbance readings (A630 nm) from the growth and proliferation assays and chemoresistance studies represent parametric, continuous data and are compiled and presented as descriptive statistics. Comparisons between treatments and between cell lines were made using two-tailed Student's t-tests and verified using Analysis of Variance (ANOVA) using Microsoft Excel (Redmond, WA, which is appropriate for analyzing parametric, continuous data.

Results

Cell Culture and STR Screening

Oral cell lines, including normal gingival fibroblasts (control) and squamous cell carcinomas, were established (Fig. 1). More specifically, normal non-cancerous HGF-1 cells were obtained, thawed, and cultured (Fig. 1A). In addition, oral squamous cell carcinoma cell lines, including SCC4, SCC9, SCC15, SCC25, and CAL27 were also obtained, thawed and cultured (Fig. 1B-1F). Verification of cell line integrity was confirmed by short tandem repeat (STR) analysis from ATCC confirming cellular identifies with 92-100% matches, as previously described (Coon *et al*., 2020; Coon and Kingsley, 2021).

Chemotherapy Resistance

To determine whether any of the oral cancer cell lines exhibited any measurable resistance to chemotherapy, Cisplatin, Fluorouracil, or 5-FU and Paclitaxel (Taxol) - the three main oral cancer therapeutic agents were tested in 96-well proliferation assays on all oral cancer cell lines (Fig. 2). These data demonstrated that each cell line exhibited distinct cell-line specific responses to each chemotherapeutic agent. For example, SCC4 cells exhibited reductions in cell growth to all three agents, ranging from -37.9% with Cisplatin, -35.2% with 5-FU, and -32.5% with Taxol. The differences between Cisplatin growth inhibition and 5-FU inhibition were not statistically significant, $p = 0.457$, which was also observed between the administration of 5-FU and Taxol, $p = 0.114$. However, there was a statistically significant difference in growth inhibition between Cisplatin and Taxol, $p = 0.0083$.

In contrast, growth inhibition among the SCC9 cells was much less significant, ranging between -18.6% with Cisplatin, -15.9% with 5-FU, and -3.3% with Taxol. The difference in growth inhibition between Cisplatin and 5-FU was not statistically significant, $p = 0.091$. However, the differences between Cisplatin and Taxol (p=0.0021) and 5-FU with Taxol ($p = 0.0086$) were statistically significant. This very large difference in responsiveness may suggest that chemo resistance to Taxol might be more prominent and specific to this cell line.

Growth inhibition among the SCC15 cells was more pronounced and also more similar, only ranging from -65.4% with Cisplatin, -68.3% with 5-FU, and -62.7% with Taxol. More detailed analysis revealed no significant differences between inhibition between Cisplatin and 5-FU ($p = 0.183$), Cisplatin and Taxol ($p = 0.464$) or 5-FU and Taxol ($p = 0.435$).

Similar to the observations made with SCC9 cells, growth inhibition among the SCC25 cells was much less significant, ranging between -11.9% with Cisplatin, -10.9 with 5-FU, and -3.6% with Taxol. The difference in growth inhibition between Cisplatin and 5-FU was not statistically significant, $p = 0.085$. However, the differences between Cisplatin and Taxol ($p = 0.031$) and 5-FU with Taxol ($p = 0.023$) were statistically significant. This very large difference in responsiveness may suggest that chemo resistance to Taxol may also be more prominent and specific to this cell line.

Finally, the observations made with CAL27 cells were similar to those made with SCC4 cells, with growth inhibition ranging between -40.1% with Cisplatin, -44.3% with 5-FU, and -40.7% with Taxol. Further analysis of these data revealed no significant differences in growth inhibition between Cisplatin and 5-FU ($p = 0.152$), 5-FU and Taxol ($p = 0.140$), or Cisplatin and Taxol ($p = 0.1001$).

To provide a differential assessment of chemotherapeutic resistance, the 96-well growth assay data were plotted against the experimental treatments, Cisplatin, 5-FU, and Taxol (Fig. 3). The graphing of these data demonstrated the stark differences among SCC9 and SCC25 cells that displayed the most robust chemoresistance among all the cell lines tested. Moreover, the similarities between SCC9 and SCC25 resistance to Cisplatin and 5-FU and the almost complete resistance to Taxol can also be more easily observed. These differences suggest that underlying mechanisms, such as differential genetic mutations or microRNA expression, may be responsible for these observations.

In addition, the similarities between SCC4 and CAL27 exhibiting moderate growth inhibition compared with the robust growth inhibition observed with SCC15 may also suggest that some underlying mechanisms, such as genetic mutations or differential microRNA expression, may be responsible for these differences.

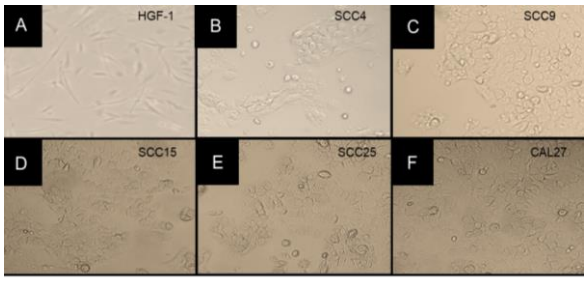
RNA extraction was performed on each cell line and assessed for purity and concentration (Table 1). These data revealed RNA concentrations within the manufacturer protocol specifications (between 100-1000 ng), which averaged 447.92 ng/uL. The purity of extracted RNA was determined by comparing the ratio of absorbance at A260 and A280 nm, which revealed that RNA purity was above 1.75 for all cell lines. In addition, secondary purity measures of phenol carryover were performed by measuring absorbance at A230, which revealed minimal contamination of this reagent.

Synthesis of cDNA from the extracted RNA was performed in preparation for qPCR screening (Table 2). This analysis revealed that cDNA concentrations were fairly consistent with an average of 1689 ng/uL - ranging from 1606.4-1802.1 ng/uL. In addition, absorbance was also measured using A 260: A 280 ratios, which revealed high DNA purity averaging 1.818-ranging from 1.80 to 1.84.

Screening of cDNA for positive control genes and microRNA expression was performed using qPCR (Fig. 4). This screening revealed all cell lines produced mRNA for the metabolic and structural genes and positive controls (GAPDH, beta-actin) with oral cancers exhibiting Ct counts between 10 - 15, which suggest both high expression and cellular abundance as expected. The positive control microRNA miR-16 was also detected from each sample, with Ct counts between 12 - 18.

In contrast, expression of miR-21 and miR-133 was restricted to the oral cancer cell lines (although expression levels varied widely), but neither miR-21 nor miR-133 was detected in the normal, non-cancerous HGF-1 cell line. Differential expression was observed among the other microRNAs with miR-27 expression observed among SCC4, SCC15, and CAL27 cells, miR-155 expression observed among SCC4, SCC9, SCC25, and CAL27 cells, and miR-135, which was observed among SCC4, SCC25 and CAL27 cells, respectively. The most limited expression was observed with miR-375, which was only observed among SCC9 and SCC25 cells.

To determine if any of the screened microRNAs identified were exported, Extracellular Vesicles (EV) and exosomes were collected for analysis (Fig. 5). The Particle Metrix - Nanoparticle Tracking Analysis or NTA was used to confirm the presence of extracellular vesicles and exosomes, which were well within the target range of 50-300 nm in size. More specifically, peak EV averages were 130.95 nm (ranging between 102.6 and 157.6 nm) with mean EV averages confirmed at 142.78 nm (ranging between 113.2 and 182 nm). These values correspond to well-established parameters and size distributions for EVs and exosomes.



Cell line	STR	Match	cell type	culturing requirements
HGF-1 (CRL-2014)	12/12	100%	normal gingival fibroblast	DMEM
CAL27 (CRL-2095)	14/15	93%	oral squamous carcinoma	DMEM
SCC25 (CRL-1628)	12/12	100%	oral squamous carcinoma	DMEM:F12
SCC15 (CRL-1623)	15/16	94%	oral squamous carcinoma	DMEM:F12
SCC9 (CRL-1629)	13/13	100%	oral squamous carcinoma	DMEM:F12
SCC4 (CRL-1624)	12/13	92%	oral squamous carcinoma	DMEM:F12

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Fig. 1: Experimental cell lines. A - F) Cultures of non-cancerous HGF-1 and oral squamous cell carcinoma cell lines, SCC4, SCC9, SCC15, SCC25, and CAL27 were established according to the supplier protocols. G) Verification by Short Tandem Repeat (STR) analysis confirmed all cell line identities with 92-100% matches

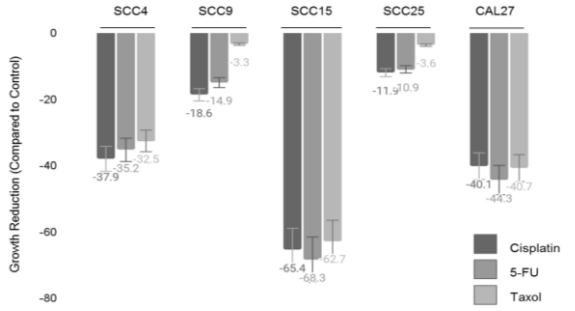

Fig. 2: Chemoresistance among oral cancer cell lines. Experimental administration of Cisplatin, Fluorouracil or 5-FU, and Paclitaxel or Taxol revealed similar ranges of growth inhibition between SCC4 and CAL27 cells, with more significant inhibition observed among SCC15 cells. Less robust inhibition was observed among SCC9 and SCC25 cells, with significantly more resistance observed in these cell lines to taxol

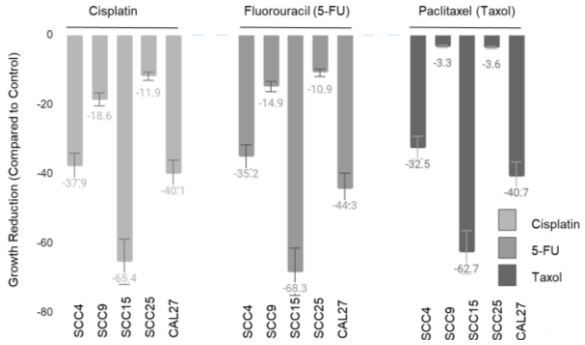

Fig. 3: Oral cancer cell line response to chemotherapy agents. Limited resistance to all three agents was observed with SCC15 cells, while moderate growth inhibition was observed with SCC4 and CAL27. More robust resistance was observed with SCC9 and SCC25 cells to Cisplatin and 5-FU with almost complete resistance to Taxol

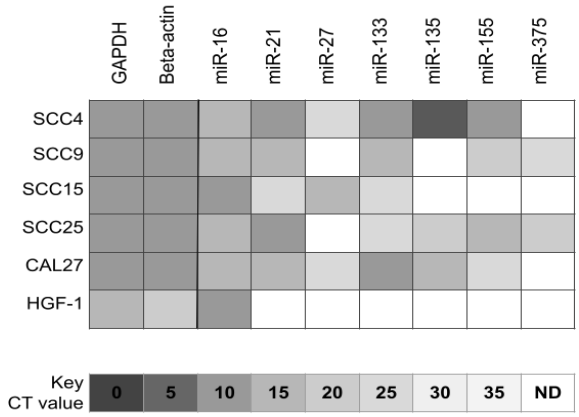

Fig. 4: qPCR screening for microRNA expression among cell lines using cellular RNA. Analysis of qPCR screening revealed positive expression for cellular genes (GAPDH, beta-actin) and miR-16 positive controls. Expression of miR-21 and miR-133 was observed in all oral cancers-although expression was varied. Differential and varied expressions of miR-27 (SCC4, SCC15, CAL27), miR-135 (SCC4, SCC25, CAL27), miR-155 (SCC4, SCC9, SCC25, CAL27), and miR-375 (SCC9, SCC25) was observed among the oral cancer cell lines only

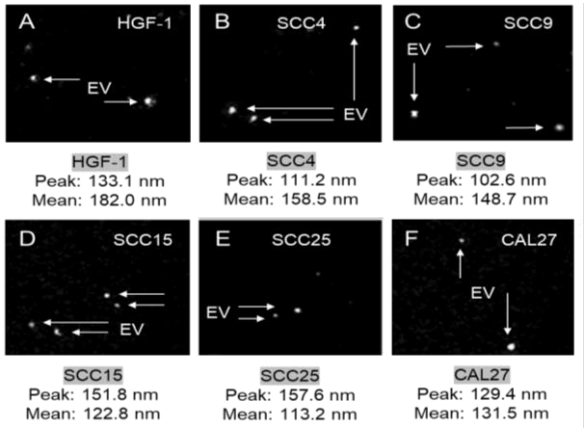

Fig. 5: Analysis of Extracellular Vesicles (EV) and Exosomes. Particle Metrix-Nanoparticle Tracking Analysis (NTA) confirmed peak and mean sizes for A) HGF-1 (133.1, 182.0 nm), B) SCC4 (111.2, 158.5 nm), C) SCC9 (102.6, 148.7 nm), D) SCCC15 151.8, 122.8 nm), E) SCC25 (157.6, 113.2 nm), and F) CAL27 (129.4, 131.5 nm)

To determine if any of the microRNAs observed from the cellular RNA screening were also exported, RNA was extracted from the EVs and exosomes and screened using qPCR (Fig. 6). This analysis revealed that miR-16 was detected from the EVs and exosomes of all cell lines, similar to the observations from the cellular microRNA screening. In addition, miR-21 and miR-133 were observed from EVs and exosomes among all of the oral cancers - but, not the HGF-1 cells, similar to the cellular microRNA expression profiles observed.

Table 1: Assessment of RNA concentration and purity

Cell line	RNA concentration [ng/uL]	RNA purity A260:A280	RNA purity A260: A230
HGF-1	482.31 ng/uL	1.81	2.07
SCC4	404.18 ng/uL	1.78	2.06
SCC9	361.15 ng/uL	1.86	2.02
SCC15	469.62 ng/uL	1.76	2.05
SCC25	484.17 ng/uL	1.81	2.15
CAL27	486.14 ng/uL	1.80	2.11
Average	447.92 ng/uL	1.80	2.07
Range	361.15-486.14 ng/uL	1.74-1.86	2.02-2.15

Table 2: Assessment of cDNA synthesis reactions

Cell line	DNA concentration [ng/uL]	DNA purity A260:A280
HGF-1	1606.4 ng/uL	1.81
SCC4	1676.7 ng/uL	1.84
SCC9	1671.8 ng/uL	1.82
SCC15	1753.2 ng/uL	1.81
SCC25	1802.1 ng/uL	1.83
CAL27	1623.9 ng/uL	1.80
Average	1689.02 ng/uL	1.818
Range	1606.4-1802.1 ng/uL	1.81-1.84

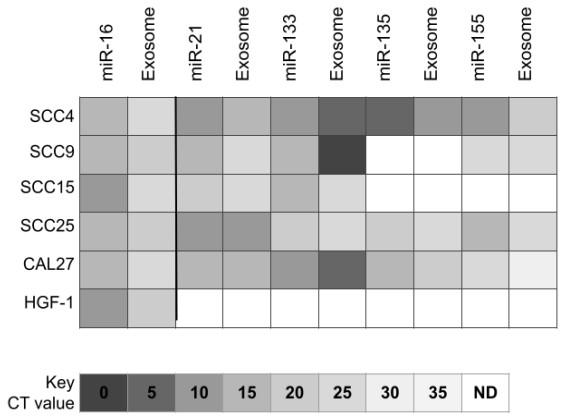

Fig. 6: qPCR screening for microRNA expression among cell lines using EV and exosomal RNA. Analysis of qPCR screening revealed miR-16 and miR-21 EV and exosomal expression matched the cellular RNA screening. Differential expression of miR-133, miR-135, and miR-155 was observed among the oral cancers, with no detection of miR-27 and miR-375 among the EVs and exosomes

Some of the differentially expressed microRNAs, such as miR-135 and miR-155 were detected from the EVs and exosomes of the corresponding cell lines that expressed these cellular microRNAs. For example, SCC4, SCC25, and CAL27 expressed miR-135 and miR-155 among the cellular RNAs and the EV and exosomal RNAs. Finally, some microRNAs were not detectable among the EVs and exosomes, such as miR-27 and miR-375.

Discussion

The primary goal of this study was to perform a targeted microRNA screening to determine whether any novel correlations with oral cancer chemotherapeutic resistance could be found. This study evaluated growth and chemotherapy resistance among some well-characterized cell lines, revealing that SCC15 exhibited relatively limited resistance to all three chemotherapeutic agents in this study (Cisplatin, 5-FU, and Taxol), while other cell lines such as SCC4 and CAL27 exhibited moderate resistance to these agents. Interestingly, SCC9 and SCC25 cells exhibited the most resistance to all three agents, with particularly robust resistance noted with Taxol. Combined with the screening results of the microRNA expression from both cellular RNA, as well as exosomes and EVs - these data confirm many previous observations, but also provide novel observations of additional microRNAs that may be associated with chemotherapeutic resistance.

For example, this study found that SCC15 exhibited the least resistance to any chemotherapeutic agents tested results that support previous observations with this cell line (Zhang *et al*., 2017). However, in addition to this observation, this study also found that SCC15 cells did not express either miR-135 or miR-155, which have both been shown to mediate chemoresistance in other oral cancer cells and models (Kirave *et al*., 2020; Zhang *et al*., 2020). This may be the first screening of these microRNAs in SCC15 cells, which may allow for a more detailed analysis of these mechanisms to follow in subsequent studies.

In addition, these results found additional cell lines that exhibited low to moderate resistance which included SCC4 and CAL27 cells as well as SCC15. This study found that differential expression of miR-27 was observed in all three of these cell lines, which supports recent observations that clinical patient samples that express miR-27 may exhibit resistance to chemotherapy or associations with poor prognosi (Liu *et al*., 2019; Momen-Heravi and Bala, 2018)

However, this may be one of the first studies to reveal this specific differential microRNA expression in combination with chemotherapy resistance in well-characterized cell lines-thereby expanding the potential to more thoroughly analyze the associated pathways and mechanisms that may be responsible for these observations in future studies.

Moreover, this study found the most robust chemotherapeutic resistance among SCC9 and SCC25 cells, which also expressed miR-375. This appears to contrast with existing studies that have suggested that miR-375 inhibits oral cancer proliferation and may increase radiosensitivity (Zhang *et al*., 2017; Xu *et al*., 2019). Some studies have also demonstrated that other activating and repressing molecules, such as LUCAT1 and SNHG17 may promote oncogenesis and progression via down-regulation of miR-375 (Zhang *et al*., 2021; Tong *et al*., 2021). However, this study also found the lack of miR-27 expression was correlated with expression of miR-375-and was specific to these cell lines only. This positive-negative association between miR-27 and miR-375 expression may provide one possible explanation for these observations, which will require further analysis and research.

Finally, this study observed that neither miR-27 nor miR-375 were found among the EVs and exosomes exported into the extracellular media of these cells. These observations may support new research studies that have suggested that both exosomal and non-exosomal microRNA may be involved in cancer progression and resistance (Nik Mohamed Kamal and Shahidan, 2020). Recent evidence has suggested that differentially expressed microRNAs and comparison with drug resistance have identified several key important regulators in ovarian cancer, such as miR-30 and miR-922 (Feng *et al*., 2019; Zhang *et al*., 2018). This principle of validating biomarkers for prognosis, treatment responsiveness, and the prognosis was first validated by using exosomal and non-exosomal microRNA expression from prostate cancer patients (Foj *et al*., 2017).

Although much remains to be discovered, some lines of evidence suggest how these microRNAs may function in other tumors (Zhang *et al*., 2021; Tong *et al*., 2021; Zhang *et al*., 2019). For example, previous research has found that miR-27 may inhibit cell migration and invasion in breast cancers and enhance the sensitivity of these cancers to chemotherapy through interactions with CREB1 and PSEN-1-suggesting that the lack of expression in some tumors may correlate with cancer progression (Song *et al*., 2021; Zhao *et al*., 2021). In addition, the most recent evidence has now suggested that miR-375 may be overexpressed in some oral cancers and functions to facilitate proliferation and progression by targeting JAK2 and PAX6 - although much remains to be discovered regarding the functional mechanisms controlling these pathways (Sun *et al*., 2021; Tong *et al*., 2021).

Conclusion

remains to be elucidated about the functional roles of miR-375 and the absence of miR-27 expression, the findings of this study suggest that these specific microRNAs may exert different and opposite effects among the cell lines observed. Future research endeavors will need to evaluate the potential role of these microRNAs not only to validate their predictive capabilities as biomarkers but also to ascertain which functional pathways may be involved in the development and progression of oral cancers.

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Author's Contributions

Brady Petersen: Responsible for methodology, investigation, formal analysis, and writing-original draft preparation.

Carl Yu, Samuel Hutchings and Connor Lemmon: Responsible for methodology, data curation, formal analysis, and writing-original draft preparation.

Katherine M. Howard and Karl Kingsley: Responsible for conceptualization, methodology, resources, data curation, formal analysis, supervision and writing review and editing.

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