DIFFERENTIAL EXPRESSION OF MICRORNA MIR-145 AND MIR-155 DOWNSTREAM TARGETS IN ORAL CANCERS EXHIBITING LIMITED CHEMOTHERAPY RESISTANCE

By

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Abstract

DIFFERENTIAL EXPRESSION OF MICRORNA MIR-145 AND MIR-155 DOWNSTREAM TARGETS IN ORAL CANCERS EXHIBITING LIMITED CHEMOTHERAPY RESISTANCE

By

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Introduction: Oral cancer remains an important issue in the United States with more than 50,000 new cases per year and almost 10,000 deaths annually [1,2]. Most of the data regarding the high rates of morbidity and mortality associated with oral cancer come from the advanced age of patients at diagnosis and the late stage of the tumor at diagnosis [3,4]. Many oral cancers (once diagnosed) are resistant to one or more chemotherapies, although more research needs to be done to determine the mechanisms of this chemotherapy resistance [5,6]. New evidence has suggested that non-coding microRNAs may play a significant role in mediating and modulating chemotherapy resistance, particularly among oral cancers [7-9]. One recent study from UNLV-SDM found that the expression of miR-145 and the lack of expression of miR-155 strongly

correlated with a lack of chemotherapy resistance, although the mechanisms responsible for this observation are yet unidentified [10].

Methods: Commercially available cell lines of oral squamous cell carcinoma (OSCC) were used in this study, including CAL27, SCC-4, SCC-9 SCC-15, and SCC-25. RNA was isolated from each of the cell lines using phenol and chloroform. The isolated RNA from the cancerous cell lines was synthesized into cDNA by reverse transcription and screened for the targets of the miRNA downstream targets of miR-145 and miR-155 using qPCR.

Results: Screening for miR-155 downstream targets revealed no expression of n=9 downstream targets, including March, IKBIP, ACT, CHAF, NPEG, FQS, CDX, JAR, or KDM. However, differential expression of n=6 downstream targets was observed with OLF, TBR, BACH, ZNF IRF, and ZIC, which were expressed in all oral cancer cell lines (CAL27, SCC25, SCC15, SCC9) except SCC4.

Screening for miR-145 downstream targets revealed no expression of n=5 downstream targets, including CLLN3, FLI, MRTF, DAB and SRGAP1. Differential expression of n=8 downstream targets was observed with ADD3, MBTD, ACE1, TRIM2, FAM135A, KCN, FSCN, and SRGAP2. However, three downstream targets were differentially expressed in SCC15 only. More specifically, KCN and SRGAP2 expression was only observed in SCC15 but not other oral cancer cell lines. In addition, FAM135A was expressed in all oral cancer cell lines with the

exception of SCC15. These data strongly suggest differential regulation of these three downstream targets among the least chemotherapy resistant oral cancer cell line SCC15.

Discussion and Conclusions: Based upon the results of this study, at least three downstream targets for miR-145 are dysregulated in oral cancers that lack chemotherapy resistance, including FAM135A (non-expressed), KCN and SRGAP2 (expressed). The potential involvement of miR-145 with these genes, such as the involvement of FAM135 and SRGAP2 with Rho GTPase signaling, and KCN involvement with potassium ion channels, must be further investigated to determine how and whether these mechanisms may be involved in the lack of chemotherapy resistance. However, none of the downstream microRNA targets for miR-155 evaluated were dysregulated in oral cancers that lack chemotherapy resistance.

Key words: Oral cancer, chemotherapy resistance, microRNA expression, qPCR screening

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Chapter 1: Introduction

Background and Significance

Oral cancer remains an important issue in the United States with more than 50,000 new cases per year and almost 10,000 deaths annually [1,2]. Most of the data regarding the high rates of morbidity and mortality associated with oral cancer come from the advanced age of patients at diagnosis and the late stage of the tumor at diagnosis [3,4]. Many oral cancers (once diagnosed) are resistant to one or more chemotherapies, although more research needs to be done to determine the mechanisms of this chemotherapy resistance [5,6]. New evidence has suggested that non-coding microRNAs may play a significant role in mediating and modulating chemotherapy resistance, particularly among oral cancers [7-9]. One recent study from UNLV-SDM found that the expression of miR-145 and the lack of expression of miR-155 strongly correlated with a lack of chemotherapy resistance, although the mechanisms responsible for this observation are yet unidentified [10].

Research Questions

Question 1. Are the identified microRNA targets for miR-145 dysregulated in oral cancers that display chemotherapy resistance?

Null hypothesis: None of the potential microRNA targets for miR-145 are dysregulated

Alternative hypothesis: One (or more) potential microRNA targets for miR-145 are dysregulated

Question 2. Are the identified microRNA targets for miR-155 dysregulated in oral cancers that display chemotherapy resistance?

Null hypothesis: None of the potential microRNA targets for miR-155 are dysregulated

Alternative hypothesis: One (or more) potential microRNA targets for miR-155 are dysregulated

Approval

The appointment of an advisory committee was submitted for approval and approved on July 28, 2022. The prospectus for this study was submitted for approval and approved on September 2, 2022. This study involved an analysis of commercially available cell lines (in vitro study). No human subjects were recruited or involved in this study.

Research Design

Commercially available cell lines of oral squamous cell carcinoma (OSCC) were used in this study. They were obtained from the American Culture Tissue Collection (ATCC; Manassas, VA, USA). The following cell lines were used: CAL27 (CRL-2095), SCC-4 (CRL-1624), SCC-9 (CRL-1629), SCC-15 (CRL-1623), and SCC-25 (CRL-1628). The cell lines were

cultured in medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin from ThermoFisher Scientific (Fair Lawn, NJ, USA) following the manufacturer's guidelines. SCC-4, SCC-9, SCC-15, and SCC-25 cells were cultured in DMEM:F12. The CAL27 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). The cell cultures were maintained in tissue-culture treated flasks in a BSL-2 incubator at 37 °C with 5% CO2. Each cell line was verified by the manufacturer using the Short Tandem Repeat (STR) technique, with a validity rate exceeding 90%, as outlined in other studies [11-13].

RNA Isolation

RNA was isolated from each of the cell lines. This involved extracting the RNA using the TRIzol reagent from Invitrogen with phenol and chloroform. The extracted lysates were transferred to sterile tubes and chloroform was added. After mixing the samples and keeping them on ice for 15 minutes, a centrifuge was used to separate the RNA-containing phase from the rest of the solution. The RNA-containing phase was transferred to a sterile tube and combined with an equal amount of isopropanol, causing the nucleic acids to precipitate. After removal of the isopropanol, the pellet was washed with ethanol, and centrifuged again. The pellet was resuspended using nuclease-free distilled water.

The concentration and quality of the isolated RNA was determined using a NanoDrop spectrophotometer. Absorbance of the RNA samples was absorbed at A260 nm and A280 nm. The absorbance values allow the calculation of the relative abundance or concentration of RNA as well as the overall quality of each sample. RNA samples with a concentration greater than 100 ng and A260:A280 ratios exceeding 1.65 were considered suitable for this analysis.

cDNA and qPCR

The isolated RNA from the cancer cell lines was synthesized into cDNA by reverse transcription using a ThermoFisher RT-PCR kit. The following steps were used: cDNA synthesis for 15 minutes at 50 °C, then enzyme deactivation for 2 minutes at 95 °C. Then, 40 cycles were repeated that consisted of 20 seconds of denaturation at 95°C, annealing for 30 seconds at varying temperatures depending on the primer, and an extension at 72 °C for 60 seconds. [14]

To amplify the potential miRNA targets with low expression levels, further processing of the cDNA was done using the TaqMan miR-Amp Reaction Mix. A mixture was prepared containing the cDNA, miR-Amp Master Mix, Primer Mix, and RNase-free water. The mixture was put in a thermal cycler, and temperature cycles were used for denaturation, annealing, and extension.

Samples that met the criteria for quantity (>10 ng) and quality A260:A280 ratio above 1.60 were screened for RNA targets of the miRNAs using qPCR. Each qPCR screening was done twice. Materials used in this process included 2X ABsolute SYBR green master mix (12.5 μL), forward and reverse primers (1.5 μL each), sample DNA (1.5 μL diluted to 1.0 ng/μL) and distilled nuclease-free water (8.0 μL). Activation (15 minutes at 95°C) was performed and followed by 40 cycles of denaturation (15 seconds at 95°C), annealing (30 seconds at various temperatures depending on each primer) and extension (30 seconds at 72°C).

Primers:

miR-145 forward: 5′-AGAGAACTCCAGCTG-3′; 15 nt, 53% GC, Tm: 56 °C

miR-145 reverse: 5′-GGCAACTGTGGGGTG-3′; 15 nt, 67% GC, Tm: 64 °C

miR-155 forward: 5′-TTAATGCTAATTGTGATAGGGGT-3′; 23 nt, 35% GC, Tm: 61 °C

miR-155 reverse: 5′-CCTATCACAATTAGCATTAATT-3′; 22 nt, 27% GC, Tm: 55 °C

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Chapter 2

Differential Expression Of MicroRNA MiR-145 and MiR-155 Downstream Targets In Oral Cancers Exhibiting Limited Chemotherapy Resistance

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Role of Authors:
KK and KMH were responsible for the overall project design. CB and TD were responsible for data generation and analysis. KK, KMH, TD and CB contributed to the writing and editing of this manuscript. All authors have read and agreed to the submitted version of the manuscript.

Abstract

Background: New evidence has suggested that non-coding microRNAs play a significant role in mediating and modulating chemotherapy resistance, particularly among oral cancers. One recent study found that the expression of miR-145 and lack of miR-155 expression strongly correlated with a limited chemotherapy resistance to Cisplatin, 5-Fluorouracil and Paclitaxel , although the mechanism(s) responsible for these observations remain unidentified.

Methods: Using commercially available cell lines of oral squamous cell carcinoma (OSCC), RNA was isolated using phenol:chloroform extraction. The isolated RNA was converted into cDNA by reverse transcription and subsequently screened for the presence or absence of downstream targets of miR-145 and miR-155 using qPCR.

Results: The expression of miR-145 downstream gene targets (n=13) were analyzed in all OSCC cells. The expression pattern of three miR-145 gene targets could be correlated to the degree of chemotherapy resistance. In the least chemotherapy resistant cells (SCC15), altered expression of KCN and SRGAP2 and the absence of FAM135A expression were observed. This differential expression was unique to the SCC15 cells and not detected in any of the other OSCC cell lines.

Conclusions: These data strongly support that differential regulation of these three downstream targets is related to the chemotoxic sensitivity of the SCC15 oral cancer cell line. The potential involvement of these targets must be further investigated to determine how and whether mechanisms of these cellular pathways may be involved in the observed lack of chemotherapy resistance. These data may be important to design targets or treatments to reduce chemotherapy resistance and improve patient treatment outcomes.

Key words: Oral cancer, chemotherapy resistance, microRNA expression, qPCR screening

Introduction

Oral cancer remains an important epidemiologic concern worldwide, with recent estimates of more than 350,000 cases diagnosed annually, resulting in nearly 200,000 deaths [1]. These high rates of oral cancer morbidity and mortality may be attributable to numerous factors, although many studies now suggest late-stage diagnosis of tumors and the advanced age of patients at the time of diagnosis are among the most impactful variables [2,3]. Although many efforts are being made to foster early detection and diagnosis, it has become evident that treatment will be needed

for most of these patients and understanding the factors that determine treatment responsiveness among these tumors becomes ever more critical [4,5].

Oral cancer is complex and often involves multiple treatment modalities including surgical resection, chemotherapy and radiation treatments [6,7]. Depending upon the size, location, and stage of the tumor, oral cancers may be subject to surgical resection structured to remove the tumor mass along with a small margin of normal tissue immediately surrounding the area of concern [8,9]. These procedures may be followed with either radiation or chemotherapy as the main types of follow-up care administered to these oral cancer patients [10,11].

Chemotherapy for oral cancer typically involves one or more of several well-known treatments, such as Cisplatin, 5-Fluorouracil (5-FU) and Paclitaxel (Taxol) [12,13]. Cisplatin functions as a cytotoxic treatment by binding to DNA within rapidly dividing cells of the tumor and forming a bond between platinum and the nitrogen atom of guanine or "G", which interferes with transcription, replication and DNA repair mechanisms [14,15]. Other treatments such as 5-Fluorouracil or 5-FU function primarily as antimetabolites, inhibiting function of the enzyme thymidylate synthase, thereby inhibiting an important step in the process of DNA synthesis in rapidly dividing cells, such as tumor cells [16,17]. In addition, chemotherapy agents such as Paclitaxel or Taxol function by binding microtubules, inducing mitotic arrest at the spindle assembly checkpoint of cell division or the G2/M transition [18-20].

Despite the varied mechanisms of action of these chemotherapy agents, many oral cancers also display significant levels of resistance to one or more of these standard treatments [21,22]. The

mechanisms proposed to explain this chemoresistance have been identified as specific allelic variations or genetic mutations that allow for metabolic reprogramming and dysregulation to bypass one or more of the chemotherapy pathways or checkpoints, as outlined previously [23,24]. However, new evidence has now suggested that non-coding microRNAs may also play an alternative and significant role in mediating and modulating chemotherapy resistance, particularly among oral cancers [25,26].

MicroRNAs are small, highly conserved, non-coding RNAs involved in the regulation of gene expression through post-transcriptional mediation, such as mRNA inhibition or negative regulation [27,28]. In fact, many studies have identified microRNA expression profiles related to many types of cancers, including lung, breast, and colorectal cancers [29-31]. Moreover, recent systematic reviews have identified microRNA expression profiles more closely associated with oral cancers through large-scale salivary biomarker screening studies [32-35].

More specifically, systematic reviews and meta-analyses have established microRNA expression profiles for oral cancers including miR-21, miR-31, miR-155 and miR-196 [36-39]. In addition, many studies have revealed that microRNA expression also functions to mediate chemotherapy resistance among oral cancers [40,41]. For example, increased tumor resistance to Cisplatin has been linked with expression of miR-21, but resistance among oral cancer has also been linked with miR-24, miR-218, and miR-629, while miR-15b, miR-27b, and miR-155 may be associated with decreased resistance to Cisplatin within these same tumors [42-47].

Recent work from this group has demonstrated expression of miR-21 and miR-365 among oral cancers, as well as confirmation of the lack of miR-27 expression among chemoresistant oral cancer cell lines [48-51].

Moreover, this most recent study found that the expression of miR-145 and the absence of miR-155 expression were also strongly correlated with a lack of chemotherapy resistance, although the mechanism(s) responsible for this observation remained unidentified [51]. The goal of this current study was to provide an evaluation of these microRNAs and their downstream targets to create a more comprehensive understanding of their potential role in the lack of chemotherapeutic resistance among oral cancers, which could provide new potential treatments and therapies [47,51 52].

Materials and Methods

Cell Lines and Culture

This study utilized commercial oral cancer cell lines, which included oral squamous cell carcinomas (OSCC) of the tongue. All cell lines were purchased from the American Tissue Culture Collection or ATCC (Manassas, VA, USA). These included SCC4, SCC9, SCC15, SCC25 and CAL27. All cells were cultured and maintained using the protocols and recommendations from the manufacturer. In brief, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum or FBS (10%) and antibiotic Penicillin-Streptomycin (1%) all from Fisher Scientific (Fair Lawn, NJ, USA) were used for CAL27 cells. All other cell lines (SCC25, SCC15, SCC9, SCC4) were maintained using DMEM:F12 with 10% FBS and 1% Penicillin-Streptomycin, all obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Catalog information for ordering, the short tandem repeat (STR) analysis for verification of cell type (>90%), and the original derivation of each cell line provided by the manufacturer were provided, as follows:

Table 1. STR cell line validation

Cell line	Media	Designation	STR analysis	Original derivation
CAL27 (CRL-2095)	DMEM	OSCC	93% match	Male, 56 years old
SCC25 (CRL-1628)	DMEM:F12	OSCC	100% match	Male, 70 years old
SCC15 (CRL-1623	DMEM:F12	OSCC	95% match	Male, 55 years old
SCC9 (CRL-1629)	DMEM:F12	OSCC	100% match	Male, 25 years old
SCC4 (CRL-1624)	DMEM:F12	OSCC	92%	Male, 55 years old

Culture of cells was facilitated using tissue culture-treated flasks and a FisherBrand Isotemp CO2 Biosafety Level 2 (BSL-2) incubator from Fisher Scientific (Fair Lawn, NJ, USA) at 37 °C, which was supplemented with additional medical-grade CO_2 at 5%.

Experimental chemotherapy agents

Experimental assays utilized commercially available chemotherapy agents, which included Paclitaxel (Taxol; NSC 125973, Molecular Weight 853.91), 5-Fluorouracil (5-FU; NSC 19893, Molecular Weight 130.08), and cis-diamminedichloroplatinum (Cisplatin; NSC 119875, Molecular Weight 300.5) all obtained from Selleck Chemical (Houston, TX, USA).

Concentrations for each chemotherapy agent used in the proliferation and growth assays were within the range of 1.0 - 10.0 ng/mL to simulate the physiologic concentrations and dosages that have been validated through previous *in vivo* bioavailability studies [53,54].

Proliferation assays

Oral squamous cell carcinoma growth under experimental (chemotherapy) and control (no treatment) conditions was performed using Corning Costar 96-well tissue culture-treated assay plates from Fisher Scientific (Fair Lawn, NJ, USA). Cells were plated at standard concentrations (1×10^5 cells/mL) and were allowed to proliferate for 24, 48 and 72 hours with and without chemotherapeutic agents to establish baseline growth and determine chemotherapeutic inhibition for each cell line. All assays were performed in triplicate and each assay was performed using n=8 wells per cell line and condition. At the conclusion of each endpoint (24 hours, 48 hours, 72 hours), cells were fixed using 10% buffered formalin prior to processing. Processing of each assay plate was performed by removing the buffered formalin and adding Gentian Violet 1% aqueous solution from Ricca Chemicals (Arlington, TX, USA). The stain was aspirated and wells were washed with 10% phosphate buffered saline (PBS) obtained from Fisher Scientific (Fair Lawn, NJ, USA). All liquid was aspirated and plates were analyzed using an ELx808 Microplate Reader from BioTek Instruments (Winsooki, VT, USA) at 630 nm.

RNA extraction

Cellular RNA was extracted from all cell lines for further screening and analysis. This process involved phenol:chloroform extraction method, utilizing the TRIzol reagent obtained from Invitrogen (Waltham, MA, USA). In brief, supernatant was removed from cells in culture and

TRIzol reagent was added to facilitate cell lysis prior to transfer into sterile microcentrifuge tubes. To each 1.0 mL of cellular lysate, 200 uL of molecular-grade Chloroform from Invitrogen (Waltham, MA, USA) was added and mixed prior to incubation on ice for 15 minutes. Each sample was then centrifuged at 12,000 x relative centrifugal force (RCF) at 4°C for 15 minutes with a 5424R Refrigerated Microcentrifuge obtained from Eppendorf (Hamburg, Germany). The upper aqueous layer containing RNA (300 uL) was then transferred to another sterile microcentrifuge tube and molecular-grade Isopropanol (300 uL) was added to precipitate nucleic acids. All samples were then centrifuged at 4°C for 10 minutes to pellet the nucleic acids. Following completion of this process, the isopropanol was removed and the nucleic acid-containing pellet was washed with ethanol prior to a final centrifugation at 4°C for 10 minutes. All remaining ethanol was removed and each pellet was resuspended using nuclease-free water. Assessment of RNA concentrations and quality were performed using the NanoDrop 2000 Spectrophotometer obtained from Fisher Scientific (Fair Lawn, NJ, USA). Relative quantification and purity were determined using absorbance readings at A260 nm and A280 nm.

cDNA synthesis

To amplify microRNA from the oral cancer cell lines, RNA was processed using the TaqMan Advanced miRNA Assay conversion kit from Applied Biosystems (Waltham, MA, USA), as previously described {51,55]. This protocol includes a poly-adenylation reaction, which involved 0.5 µL 10X poly(A) buffer, 0.5 µL ATP (adenosine triphosphate), 0.3 µL poly(A) enzyme, and 1.7 µL RNase-free water added to each of the 96-wells in a qPCR reaction plate with 2.0 µL of RNA extracted from each cell line. The poly-adenylation reaction was performed using the

manufacturer recommended protocol of 37 °C for 45 minutes, followed by 65 °C for 10 minutes in a Mastercycler gradient thermal cycler from Eppendorf (Hamburg, Germany).

Following the completion of the poly-adenylation reaction, the adaptor ligation reaction was immediately performed using 3.0 µL 5X DNA ligase buffer, 4.5 µL RNase-free water added to each of 50% PEG (polyethylene glycol) 8000, 0.6 µL 25X ligation adaptor, 1.5 µL RNA ligase, and 0.4 µL RNase-free water added to each of the 96-wells containing the completed poly-adenylation reaction in the qPCR reaction plate. The adaptor ligation reaction was performed using the manufacturer recommended protocol of 16 °C for 60 minutes.

Following the adaptor ligation reaction, the reverse transcription (RT) reaction was immediately performed using 6.0 µL 5X RT buffer, 1.2 µL dNTP mix, 1.5 µL 20X universal RT primer, 3.0 µL 10 X RT enzyme mix, and 3.3 µL RNase-free water added to each of the 96-wells containing the adaptor ligation reaction. The RT reaction was performed using the manufacturer recommended protocol of 42 °C for 15 minutes, followed by 85 °C for an additional five minutes.

The final step reaction in this protocol was the amplification of the cDNA using the TaqMan miR-Amp Reaction Mix, which included 25.0 µL 2X miR-Amp Master Mix, 2.5 µL 20X Primer Mix and nuclease, 17.5 µLRNase-free water and 5.0 uL of the RT reaction product. The amplification reaction was performed using the manufacturer recommended protocol of 95 °C for five minutes, followed by 14 cycles of 95 °C for three seconds, extension at 60 °C for 30 seconds, and a stop reaction at 99 °C for ten minutes.

qPCR screening

Screening of the cDNA for microRNA expression was completed using the SYBR Green qPCR Master Mix from ThermoFisher Scientific (Fair Lawn, NJ, USA) using the manufacturer recommended protocols. In brief, each reaction was prepared with 12.5 uL Absolute SYBR Green, 1.75 uL forward and reverse primers, 7.5 uL nuclease-free water and 1.5 uL sample cDNA for a total reaction volume of 25 uL. Thermocycle reactions were performed using the QuantStudio Real-Time Polymerase Chain Reaction (PCR) system from Applied Biosciences (Waltham, MA, USA) with 95 °C denaturation for 15 seconds, annealing at each primer pair specific temperature, and 72 °C final extension for 30 seconds. The validated primer sets (Table 2) included [51,55]:

Table 2. Validated qPCR primers

Positive control primers		
GAPDH forward	5′ATCTTCCAGGAGCGAGATCC-3′	Tm: 66 °C
GAPDH reverse	5′ACCACTGACACGTTGGCAGT-3′	Tm: 70 °C
Beta-actin forward	5′-GTGGGGTCCTGTGGTGTG-3′	Tm: 69 °C
Beta-actin reverse	5′-GAAGGGGACAGGCAGTGA-3′	Tm: 67 °C

microRNA primers		
miR-16 forward	5′-TAGCAGCACGTAAATATTGGCG-3	Tm: 65 °C
miR-16 reverse	5′-TGCGTGTCGTGGAGTC-3′	Tm: 65 °C
miR-21 forward	5′-GCCACCACACCAGCTAATTT-3′	Tm: 66 °C
miR-21 reverse	5′-CTGAAGTCGCCATGCAGATA-3′	Tm: 65 °C
miR-27 forward	5′-ATATGAGAAAAGAGCTTCCCTGTG-3’	Tm: 61 °C
miR-27 reverse	5′-CAAGGCCAGAGGAGGTGAG-’3′	Tm: 67 °C
miR-124 forward	5′-TTCACAGCGGACCTTGA-3′	Tm: 64 °C
miR-124 reverse	5′-GAACATGTCTGCGTATCTC-3′	Tm: 60 °C
miR-125 forward	5′-GCCCTCCCTGAGACCTCAA-3′	Tm: 69 °C
miR-125 reverse	5′-GTGCAGGGTCCGAGGT-3′	Tm: 68 °C
miR-133 forward	5′-CCGGTTAACTCGAGCTCTGTGAGAG-3′	Tm: 71 °C
miR-133 reverse	5′-CTAGCTAGGAATTCTGTGACCTGTG-’3′	Tm: 66 °C
miR-135 forward	5′-CGATATGGCTTTTTATTCCTA -3′	Tm: 56 °C
miR-135 reverse	5′-GAGCAGGGTCCGAGGT -3′	Tm: 67 °C

miR-140 forward	5′-GGGCAGTGGTTTTACCCTA -3′	Tm: 64 °C
miR-140 reverse	5′-CAGTGCGTGTCGTGGAGT -3′	Tm: 68 °C
miR-143 forward	5′-AGTGCGTGTCGTGGAGTC-3′	Tm: 68 °C
miR-143 reverse	5′-GCCTGAGATGAAGCACTGT-3′	Tm: 65 °C
miR-145 forward	5′-AGAGAACTCCAGCTG-3′	Tm: 56 °C
miR-145 reverse	5′-GGCAACTGTGGGGTG-3′	Tm: 64 °C
miR-152 forward	5′-GGTTCAAGACAGTACGTGACT-3′	Tm: 64 °C
miR-152 reverse	5′-CCAAGTTCTGTATGCACTGA-3′	Tm: 62 °C
miR-155 forward	5′-TTAATGCTAATTGTGATAGGGGT-3′	Tm: 61 °C
miR-155 reverse	5′-CCTATCACAATTAGCATTAATT-3′	Tm: 55 °C
miR-210 forward	5′-CATAGATAGCCACTGCCCACA-3′	Tm: 67 °C
miR-210 reverse	5′-GTGCAGGGTCCGAGGTATTC-3′	Tm: 68 °C
miR-218 forward	5′-TCGGGCTTGTGCTTGATC T-3′	Tm: 65 °C
miR-218 reverse	5′-GTGCAGGGTCCGAGTG-3’	Tm: 66 °C
miR-221 forward	5′-CCCAGCATTTCTGACTGTTG-3′	Tm: 64 °C
miR-221 reverse	5′-TGTGAGACCATTTGGGTGAA-3′	Tm: 64 °C

miR-222 forward	5′-CGCAGCTACATCTGGCTACTG-3′	Tm: 68 °C
miR-222 reverse	5′-GTGCAGGGTCCGAGGT-3′	Tm: 68 °C
miR-224 forward	5′-GCGAGGTCAAGTCACTAGTGGT-3′	Tm: 69 °C
miR-224 reverse	5′-CGAGAAGCTTGCATCACCAGAGAACG-3′	Tm: 72 °C
miR-320 forward	5′-AACGGAGAGTTGGGTCGAAA-3′	Tm: 66 °C
miR-320 reverse	5′-TTGCCTCTCAACCCAGCTTT-3′	Tm: 67 °C
miR-365 forward	5′-ATAGGATCCTGAGGTCCCTTTCGTG-3′	Tm: 70 °C
miR-365 reverse	5′-GCGAAGCTTAAAAACAGCGGAAGAGTTTGG-3′	Tm: 72 °C
miR-375 forward	5′-GGCTCTAGAGGGGACGAAGC-3′	Tm: 70 °C
miR-375 reverse	5′-GGCAAGCTTTTTCCACACCTCAGCCTTG-3′	Tm: 74 °C
miR-424 forward	5′-AGGACGAAACACCCCCTATTCCTTGC-3′	Tm: 73 °C
miR-424 reverse	5′-TAATGGATCCGAATACCTGCTCCTGA-3′	Tm: 69 °C
miR-494 forward	5′-GAAGATCTACGTCTGGTCTACCCAGTGC-3′	Tm: 72 °C

miR-494 reverse	5′-GGGGTACCACCGAGAGTGGAGCCGGCAA-3′	Tm: 82 °C
miR-654 forward	5′-GGGATGTCTGCTGACCA-3′	Tm: 64 °C
miR-654 reverse	5′-CAGTGCGTGTCGTGGA-3′	Tm: 65 °C
miR-720 forward	5′-GCGTGCTCTCGCTGGGG-3′	Tm: 73 °C
miR-720 reverse	5′-GTGCAGGGTCCGAGGT-3′	Tm: 68 °C
miR-1246 forward	5′-TGAAGTAGGACTGGGCAGAGA-3′	Tm: 67 °C
miR-1246 reverse	5′-TTTGGGTCAGGTGTCCACTC-3′	Tm: 67 °C
Downstream primers, miR-145		
FSCN1 forward (TS 100)	5′-CCAGGGTATGGACCTGTCTG-3′	Tm: 65°C
FSCN1 reverse (TS 100)	5′-GTGTGGGTACGGAAGGCAC-3′	Tm: 65°C
ABHD17C forward (TS 100)	5′-CTACTCGGGATACGGCGTCA-3′	Tm: 65°C
ABHD17C reverse (TS 100)	5′-AGAGGATAATGTTCTCGGGACTC-3′	Tm: 63°C
FLI forward (TS 100)	5′-CAGCCCCACAAGATCAACCC-3′	Tm: 65°C

FLI reverse (TS 100)	5′-CACCGGAGACTCCCTGGAT-3′	Tm: 65°C
MRTFB forward (TS 100)	5′-ATGGATCACACAGGGGCGATA-3	Tm: 63°C
MRTFB reverse (TS 100)	5′-CCGCTGGGCTCTTCAAAGG-3′;	Tm: 65°C
DAB2 forward (TS 100)	5′-GTAGAAACAAGTGCAACCAATGG-3′	Tm: 61°C
DAB2 reverse (TS 100)	5′-GCCTTTGAACCTTGCTAAGAGA-3′	Tm: 61°C
SRGAP1 forward (TS 100)	5′-ACCCCGAGCCGATTCAAGA-3′	Tm: 62°C
SRGAP1 reverse (TS 100)	5′-GAACTCGCATCTCCGTTTGCT-3′	Tm: 63°C
SRGAP2 forward (TS 100)	5′-TGAAGGAGAAAGCGTCAAGCC-3′	Tm: 62°C
SRGAP2 reverse (TS 100)	5′-AAGGTCAGATAGGTCATGGATGT-3′	Tm: 61°C
CLCN3 forward (TS 99)	5′-GGAGGCAGCATTAACAGTTCT-3′	Tm: 61°C
CLCN3 reverse (TS 99)	5′-TCGCACCCAATCAATAGTATGGA-3′	Tm: 61°C
MBTD1 forward (TS 99)	5′-GGCATGGCTACCTGTGAGATG-3′	Tm: 65°C
MBTD1 reverse (TS 99)	5′-GGCCAAAATGCTTGCCTTCT-3′	Tm: 61°C
FAM135A forward (TS 99)	5′-AGTAGCCGAACATTGAAGCTG-3′	Tm: 61°C
FAM135A reverse (TS 99)	5′-TGGCTGGTGTAGTGCAACC-3′	Tm: 62°C
ABCE1 forward (TS 99)	5′-GGAATGCAAAAAGAGTTGTCCTG-3′	Tm: 61°C

ABCE1 reverse (TS 99)	5′-CGAGGGATAGGCAACCTGTG-3′	Tm: 65°C
KCNA4 forward (TS 99)	5′-GTACCTCCCATGACCCTCAGA-3′	Tm: 65°C
KCNA4 reverse (TS 99)	5′-CTGCCGGTAGTGGGCTTTC-3′	Tm: 65°C
ADD3 forward (TS 99)	5′-CCAGCCAAGGCGTGATTAC′	Tm: 62°C
ADD3 reverse (TS 99)	5′-TGAAGTCTTGTCGTAGATCAGGA-3′	Tm: 61°C
TRIM2 forward (TS 99)	5′-TGCGCCAGATTGACAAGCA′;	Tm: 60°C
TRIM2 reverse (TS 99)	5′-GCACCTCTCGCAGAAAGTG-3′	Tm: 62°C
Downstream primers, miR-155		
ZNF652 forward (TS 99)	5′-GCTGGTTGAAAACTGTGCTGT-3′	Tm: 61°C
ZNF652 reverse (TS 99)	5′-GAAGATGGCACTTGACCACGA-3′	Tm: 63°C
ZIC3 forward (TS 99)	5′-CGGCGCACGATCTATCTTCAG-3′	Tm: 65°C
ZIC3 reverse (TS 99)	5′-TGCGGAACAGAAACTCGC-3′	Tm: 62°C
BACH1 forward (TS 99)	5′-TCTGAGTGAGAACTCGGTTTTTG-3′	Tm: 61°C
BACH1 reverse (TS 99)	5′-CGCTGGTCATTAAGGCTGAGTCC-3′	Tm: 63°C
JARID2 forward (TS 99)	5′-ACCAGTCTAAGGGATTAGGACC-3′	Tm: 63°C

JARID2 reverse (TS 99)	5′-TGCTGGGACTATTCGGCTGA-3′	Tm: 62°C
KDM5B forward (TS 97)	5′-CCATAGCCGAGCAGACTGG-3′	Tm: 65°C
KDM5B reverse (TS 97)	5′-GGATACGTGGCGTAAAATGAAGT-3′	Tm: 61°C
TBR1 forward (TS 97)	5′-GCAGCAGCTACCCACATTCA-3′	Tm: 62°C
TBR1 reverse (TS 97)	5′-AGGTTGTCAGTGGTCGAGATA-3′	Tm: 61°C
IRF2-BP2 forward (TS 97)	5′-CCCATGACTCCTACATCCTCTT-3′	Tm: 63°C
IRF2-BP2 reverse (TS 97)	5′-GAGGGCGGACTGTTGCTATTC-3′	Tm: 65°C
OLFML3 forward (TS 97)	5′-TCCTTTTGTCATGGTCGGGAC-3′	Tm: 63°C
OLFML3 reverse (TS 97)	5′-TAAAGCAGCTAGTCGGCGTTC-3′	Tm: 63°C
MPEG1 forward (TS 96)	5′-CGGCAGCATGGGCTAAATCA-3′	Tm: 62°C
MPEG1 reverse (TS 96)	5′-TGTCCACATTCCGCAGATTGT-3′	Tm: 61°C
CDX1 forward (TS 96)	5′-GGTGGCAGCGGTAAGACTC-3′	Tm: 65°C
CDX1 reverse (TS 96)	5′-TGTAACGGCTGTAATGAAACTCC-3′	Tm: 61°C
ACTL7A forward (TS 96)	5′-TGGGTCCGCCATACGAGTT-3′	Tm: 62°C
ACTL7A reverse (TS 96)	5′-GTCCACGACCACTGCTTTG-3′	Tm: 62°C
MARCH1 forward (TS 96)	5′-CACTGGGACACTGCGCTTT-3′	Tm: 62°C

MARCH1 reverse (TS 96)	5′-TCACAGCAGCGTGTATCTGAG-3′	Tm: 63°C
FOS forward (TS 96)	5′-CCGGGGATAGCCTCTCTTACT-3	Tm: 65°C
FOS reverse (TS 96)	5′-CCAGGTCCGTGCAGAAGTC-3′	Tm: 65°C
IKBIP forward (TS 96)	5′-GCTCATCTAAAGCGTCTACAGG-3′	Tm: 63°C
IKBIP reverse (TS 96)	5′-AAGCGTCGTCAGACTGTTGTT-3′	Tm: 61°C
CHAF1A forward (TS 96)	5′-AGCCCGTCTGCCGTTTAAG-3′	Tm: 62°C
CHAF1A reverse (TS 96)	5′-AGAAGTACCCTGATCGTCTGAC-3′	Tm: 63°C

Results

The oral cancer cell lines were grown with and without the addition of the chemotherapy agents (Figure 1). More specifically, the addition of Cisplatin, 5-FU and Taxol inhibited the growth of all oral cancer cell lines - although these effects exhibited extensive variability. For example, the cell lines SCC25 and SCC9 exhibited the most resistance (and the least inhibition to growth) against all three chemotherapeutic agents, ranging between -3.3% to -18.6%. Other cell lines, such as SCC4 and CAL27, exhibited less resistance and moderate inhibition of cell growth ranging between -32.5% to -44.3%. However, one cell line in particular, SCC15, exhibited the

least resistance and the most inhibition to growth to all three chemotherapy agents, which ranged from -62.7% to -68.3%.

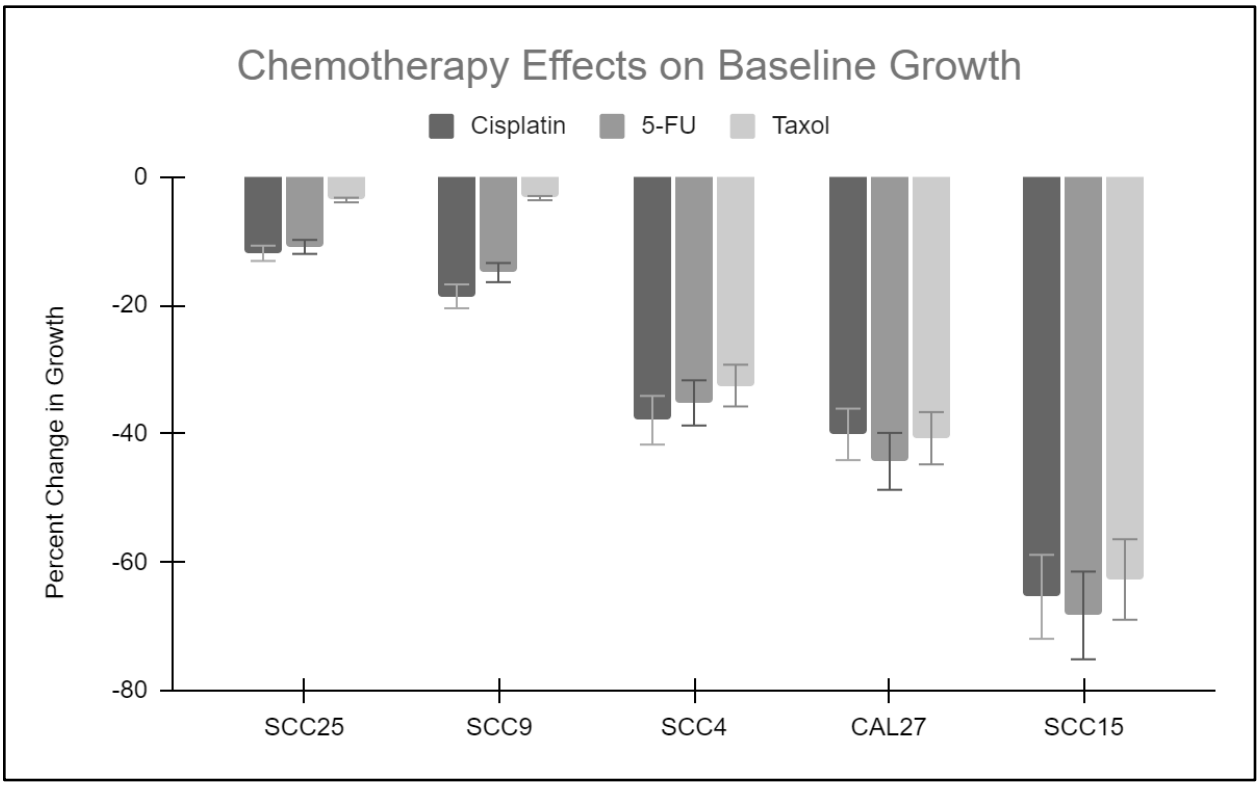

Figure 1. Comparison of baseline (control) growth with experimental treatment among oral cancer cell lines. The most chemoresistant (least inhibited) cell lines to all three chemotherapy agents (Cisplatin, 5-FU, Taxol) included SCC25 and SCC9 with moderate inhibition observed among SCC4 and CAL27 cells. The least chemoresistant (most inhibited) cell line was SCC15.

To determine any differences in expression that may modulate the observed differences in chemotherapy resistance, RNA was extracted from all cell lines (Table 3). These data demonstrated the successful isolation of RNA from all cell lines, which averaged 454.6 +/- 44.6 ng/uL and ranged from 422 to 492 ng/uL. Purity of RNA, determined by the ratio of absorbance at A260 to A280, averaged 1.79 among the cancer cell lines with a range between 1.77 and 1.81. Synthesis of cDNA from the isolated RNA was completed, which demonstrated concentrations that averaged 1526 +/- 53.6 with a range between 1499 and 1552 ng/uL. Purity of cDNA averaged 1.84, which ranged between 1.81 and 1.88.

Table 3. RNA and cDNA analysis

Cell line	**RNA concentration**	**RNA purity [A260:A280 ratio]**	**cDNA concentration**	**cDNA purity [A260:A280 ratio]**
SCC4	422 +/- 38 ng/uL	1.81	1552 +/- 57 ng/uL	1.84
SCC9	461 +/- 41 ng/uL	1.77	1499 +/- 61 ng/uL	1.81
SCC15	492 +/- 44 ng/uL	1.79	1523 +/- 52 ng/uL	1.82
SCC25	443 +/- 49 ng/uL	1.80	1531 +- 51 ng/uL	1.88
CAL27	455 +/- 51 ng/uL	1.81	1528 + 47 ng/uL	1.86
Average Range	454.6 +/- 44.6 422 - 492 ng/uL	1.79 1.77 - 1.81	1526 +/- 53.6 1499 - 1552 ng/uL	1.84 1.81 - 1.88

To confirm and verify the results of the previous studies, qPCR screening for expression of microRNAs was performed for all oral cancer cell lines (Figure 2). Several microRNAs were found to be expressed in all oral cancers to varying degrees, including miR-16 (positive control),

miR-21, miR-125, miR-133, miR-365, miR-720 and miR-1246. In addition, several microRNAs were not found to be expressed among any of the oral cancer cell lines, which included miR-140, miR-152, miR-218, miR-221, and miR-224.

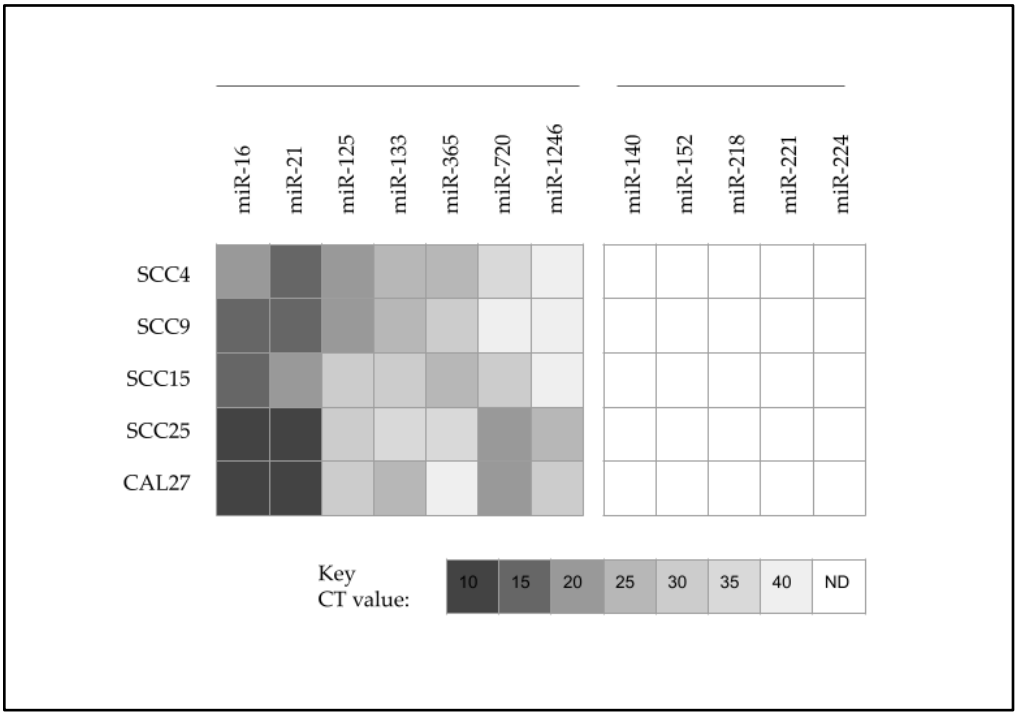

Figure 2. Analysis of qPCR screening for oral cancer microRNA expression. All oral cancer cell lines expressed miR-16, miR-21, miR-125, miR-133, miR-365, miR-720 and miR-1246, while no oral cancer cell lines expressed miR-140, miR-152, miR-218, miR-221, or miR-224.

Further analysis of the qPCR screening results revealed that several microRNAs were found to be differentially expressed in some, but not all, oral cancer cell lines (Figure 3). For example, miR-124 and miR-210 were expressed only among SCC4 cells, while miR-143 was observed only among CAL27 cells. Most microRNAs were expressed in at least two or three oral cancer cell lines, including miR-27, miR-135, miR-222, miR-320, miR-375, miR-424 niR-494, and miR-654. However, two microRNAs were differentially expressed among SCC15 cells only, which included miR-145 that was only observed among SCC15 cells and miR-155 that was observed in all other cell lines except SCC15 cells.

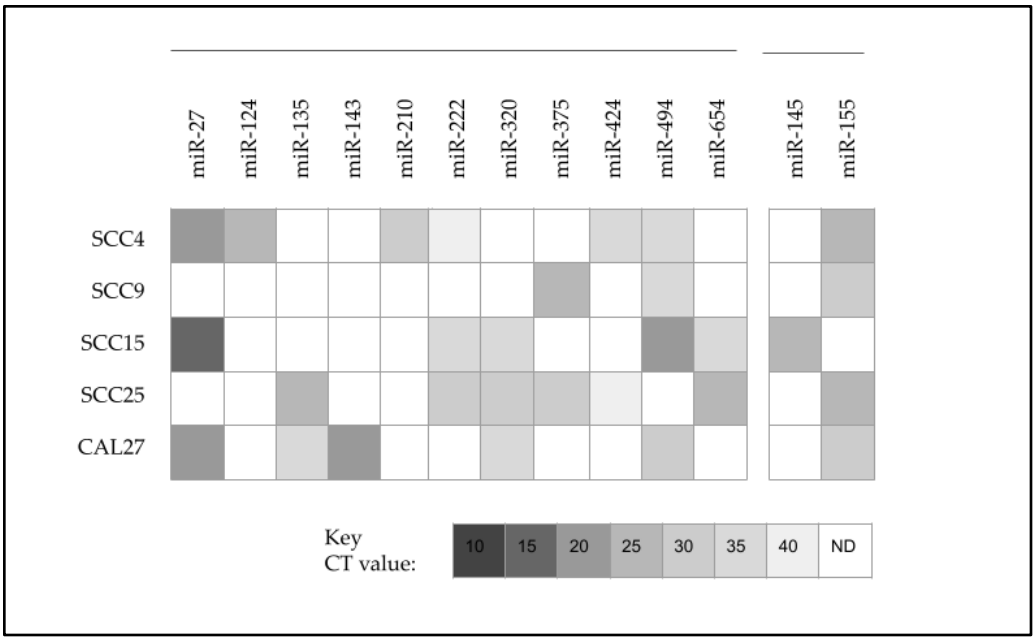

Figure 3. Differential expression of microRNAs among oral cancers. Differentially expressed microRNAs included miR-27, miR-124, miR-135, miR-143, miR-210, miR-222, miR-320, miR-375, miR-424 miR-494, and miR-654. Differentially expressed in SCC15 included miR-145 (only expressed among SCC15 cells) and miR-155 (expressed in all other cell lines except SCC15).

To more closely evaluate the potential relationship between miR-145 expression and chemoresistance of SCC15 cells, downstream targets of miR-145 were identified and screened (Figure 4). This analysis revealed that in addition to the positive control GAPDH, all oral cancers expressed the miR-145 downstream targets MBTD1 and FSCN1. In addition, none of the oral cancers expressed CLCN3, FLI-1, MRTF, DAB, SRGAP1, or ABHD17C. However, differential expression was observed with TRIM2, ADD3 and ABCE1 among some of the oral cancer cell

lines. Moreover, SCC15-specific expression was observed with KCNA4 and SRGAP2 and lack of expression among SCC15 cells of FAM135A, which was expressed in all other oral cancer cell lines.

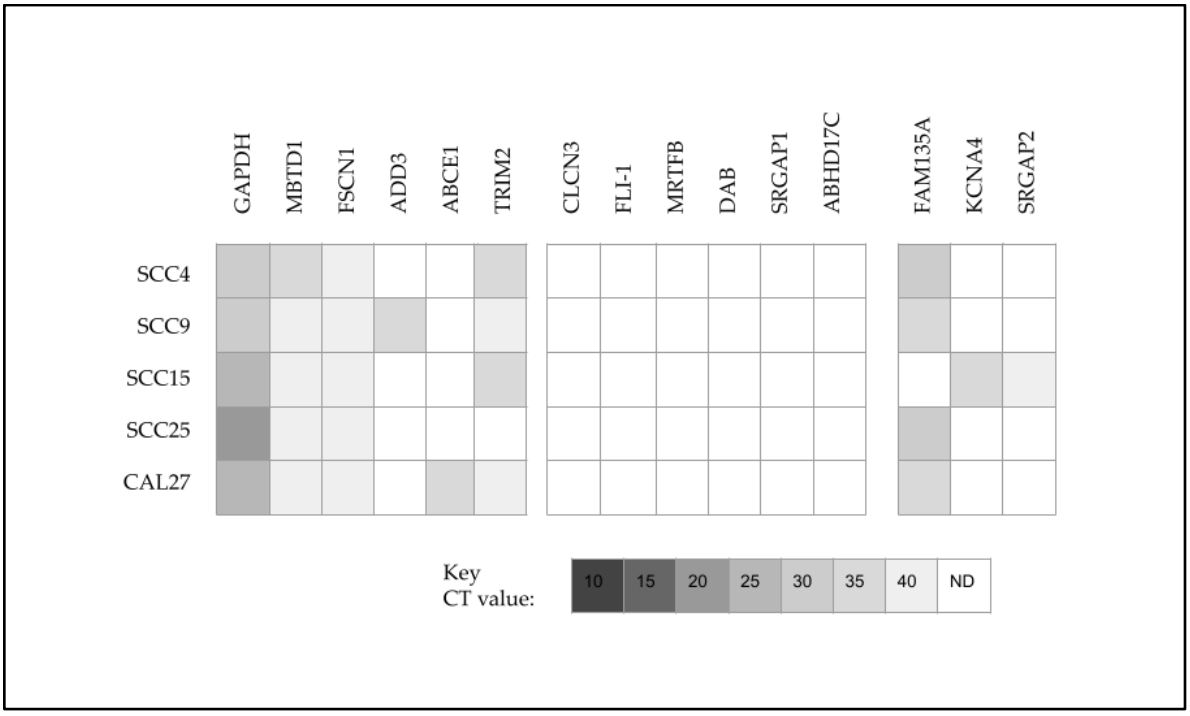

Figure 4. Screening and analysis of miR-145 downstream targets. All cell lines expressed MBTD1 and FSCN1, while none expressed CLCN3, FLI-1, MRTFB, DAB, SRGAP1, or ABHD17C. Differential expression was observed with TRIM2, ADD3 and ABCE1 with SCC15-specific expression observed with KCNA4, SRGAP2, and FAM135A.

To more closely evaluate the potential relationship between the lack of miR-155 expression and chemoresistance of SCC15 cells, downstream targets of miR-155 were also identified and screened (Figure 5). This analysis revealed that in addition to the positive control GAPDH, all oral cancers (except SCC4) expressed the miR-155 downstream targets OLFML3, TBR1, BACH1, ZNF652, IRF2-BP2 and ZIC3. In addition, none of the oral cancers expressed MARCH1, IKBIP, ACTL7A, CHAF1A, MPEG1, FOX, CDX1, JARID2 or KDM5B. No differential or SCC15-specific expression was observed among any of the miR-155 downstream targets analyzed.

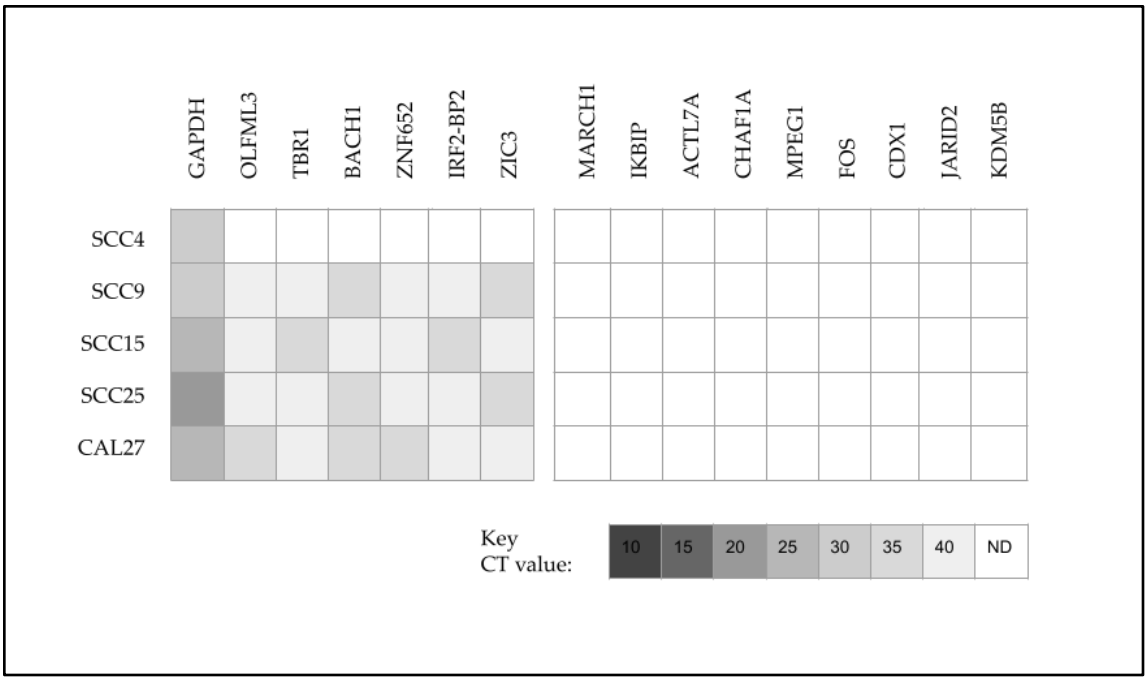

Figure 5. Screening and analysis of miR-155 downstream targets. All cell lines (except SCC4) expressed OLFML3, TBR1, BACH1, ZNF652, IRF2-BP2 and ZIC3, while none of the oral cancers expressed MARCH1, IKBIP, ACTL7A, CHAF1A, MPEG1, FOX, CDX1, JARID2 or KDM5B. No differential or SCC15-specific expression was observed among any of the miR-155 downstream targets analyzed.

Discussion

The primary objective of this current study was to provide an evaluation of the specific microRNA expression profile of the oral cancer cell line SCC15 lacking chemotherapeutic resistance, which may provide new potential insights into potential treatments and therapies. These results confirmed the expression of miR-145 among this chemosensitive cell line previously reported from this group [51]. This supports other research that demonstrates low or

lack of miR-145 expression was associated with oral cancer diagnosis and progression, while miR-145 expression correlated with improved prognosis and increased survival [32,40].

In fact, previous research has demonstrated that increased levels of miR-145 negatively correlated with oral cancer progression and may, in fact, function as an intermediary tumor suppressor [56-58]. Some evidence has suggested that miR-145 may function as a primary, direct tumor suppressor in other cancers and may function similarly to inhibit c-myc and CDK6 in oral cancers [59,60]. These mechanisms appear to support many other studies that have demonstrated that lack of miR-145 expression was associated with oral cancer progression both in vitro and in vivo [61,62].

The importance of miR-145 suppression becomes apparent as more and more overlapping mechanisms to suppress miR-145 activity are discovered, including the activity of circular RNAs, such as circ_ZNF236, circ_005063, and circ_000199 [63,64]. This research has demonstrated that other circular RNAs such as circ_GOLPH3 and circ_0001461 function to inhibit miR-145 as well as to inhibit additional downstream targets, such as KDM2 and NFkB [65,66]. Finally, many other circular RNAs, including circ_0058063 and circ_0033144 may function in concert with additional axis factors to inhibit miR-145 while upregulating other downstream targets, such as SERPINE1 and LASP1 [67,68].

The results of this current study may be the first to demonstrate the expression of miR-145 correlated with the lack of expression in the predicted downstream target FAM135A, which was expressed in the other chemoresistant cell lines and has been recently demonstrated to function in

lipid metabolism within other cancers such as breast and pancreatic cancers [69,70]. Moreover, this study also demonstrated the association between miR-145 expression and positive expression of the potassium voltage-gated channel protein KCNA4, which was also recently identified in a genome-wide differential expression study of renal cell carcinomas [71]. Finally, this study found miR-145 expression positively associated with SRGAP2 expression, which is a Rho GTPase-activating protein originally identified as regulating neuronal migration and differentiation - but more recently has been identified as a potential chemoregulatory modulator in hepatocellular carcinomas and colorectal cancers [72-74].

Although this study found no downstream targets of miR-155 (expressed in all of the chemoresistant cell lines) that were differentially expressed, it is clear that the lack of miR-155 expression among the chemosensitive cell line SCC15 is significant as this has been identified by other studies as a direct activator of additional downstream targets, such as the anti-apoptosis regulator BCL6 and pro-cell cycle regulator Cyclin D2 [75,76]. In addition, many studies have confirmed miR-155 expression may directly contribute to chemotherapy resistance to 5-FU and Cisplatin among oral cancers through additional pathway modulation, such as TP53INP1 [77-79]. Thus, continued research to confirm the lack of miR-155 expression among chemosensitive oral cancers may also help the understanding and delineation of which factors may be critical for designing treatments and therapies that increase effectiveness and efficacy.

Conclusions

The results of this study strongly support that differential regulation of key microRNAs, such as miR-145 and miR-155 may be functionally related to the chemotoxic sensitivity of the SCC15 oral cancer cell line. The potential involvement of specific downstream targets of miR-145, including FAM135A, KCNA4 and SRGAP2 must be further investigated to determine how and whether mechanisms of these cellular pathways may be involved in the observed lack of chemotherapy resistance. These data may be important to design future targets, therapies or treatments to reduce oral cancer chemotherapy resistance and improve patient treatment outcomes.

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Chapter 3

Summary and Conclusions:

This study sought to investigate the finding that oral cancers that do not display chemotherapy resistance (SCC15) also display differential microRNA expression. For example, miR-145 is expressed by SCC15 and no other commercially available oral cancer cell lines. Alternatively, miR-155 is not expressed by SCC15 and is expressed by all the other commercially available oral cancer cell lines.

The downstream targets of miR-145 and miR-155 were then evaluated and analyzed for this study. These data demonstrated that some miR-145 downstream targets were differentially expressed in SCC15 cells, while none of the miR-155 downstream targets were. More specifically, KCNA4 and SRGAP2 were expressed by only SCC15 cells, whereas FAM135A was not expressed by SCC15 cells but was observed among all other oral cancer cell lines.

These data strongly suggest that one or more of these downstream targets may play a critical role in mediating or modulating the lack of chemotherapy resistance observed among the SCC15 cell line. Further investigation of the function of these downstream targets is warranted.

Research Question 1. Are the identified microRNA targets for miR-145 dysregulated in oral

cancers that display chemotherapy resistance?

Null hypothesis: None of the potential microRNA targets for miR-145 are dysregulated

Alternative hypothesis: One (or more) potential microRNA targets for miR-145 are dysregulated

Based upon these results the null hypothesis can be rejected and the **alternative hypothesis** can be **accepted**.

Research Question 2. Are the identified microRNA targets for miR-155 dysregulated in oral cancers that display chemotherapy resistance?

Null hypothesis: None of the potential microRNA targets for miR-155 are dysregulated

Alternative hypothesis: One (or more) potential microRNA targets for miR-155 are dysregulated

Based upon these results the **null hypothesis** can be **accepted** and the alternative hypothesis can be rejected.

Limitations and Recommendations:

This study relied on commercially available oral cancer cell lines. One recommendation for future studies would be the validation of these results using primary tumors or patient explants. Another recommendation might be the experimental administration of miR-145 among other oral cancer cell lines to determine if this is sufficient to induce chemotherapy sensitivity. Alternatively, the blocking of miR-155 expression among other oral cancer cell lines may help to determine if this is sufficient to induce chemotherapy sensitivity. It might be necessary to complete both objectives concurrently, but such a results would be a significant finding that could alter how oral cancers could be treated in the near future.

Appendix A

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I, **Karl Kingsley,** holder of copyrighted material entitledDifferential Expression Of MicroRNA MiR-145 and MiR-155 Downstream Targets In Oral Cancers Exhibiting Limited Chemotherapy Resistance, authored by **Conner Belnap, Tyler Divis, Karl Kingsley, and Katherine M. Howard** originally submitted to **MDPI *Internal Journal of Molecular Sciences*, January 2024**, hereby give permission for the author to use the above described material in total or in part for inclusion in a Master's thesis at the University of Nevada, Las Vegas.

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