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Anti-Cancer Drugs Reactivate Tumor Suppressor miR-375 Expression in Tongue Cancer Cells

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ABSTRACT
Head and neck cancer is one of the deadliest malignant diseases and chemotherapy is a common treatment option. Despite the development of chemotherapies for several decades, how these drugs affect the dynamics of gene regulation is still largely unknown. In our previous study, miR-375 was shown to be underexpressed in oral cancers and thus unable to serve as a tumor suppressor microRNA to regulate certain putative oncogenes. In this study, we found that common anti-cancer drugs reactivated miR-375 in tongue cancer cells. Incubation of tongue cancer cells CAL 27 and SCC-25 in medium containing doxorubicin, 5-fluorouracil, trichostatin A, or etoposide significantly increased the expression of miR-375 and its primary transcript pri-miR-375. The dose- and time-dependent effects of doxorubicin in CAL 27 were demonstrated by miR-375 increases in response to the drug. Significant suppression of pri-miR-375 expression was observed in human tongue cancer specimens and this decrease was more prominent in advanced stage tumors. Bioinformatics from four publicly available mRNA microarray data sets suggested that these candidate miR-375 targets are mainly involved in cancer biology, indicating that these targets are likely to be suppressed via miR-375 due to the treatment with these drugs. Together, our data suggest that the four anti-cancer drugs examined in this study induce the expression of tumor suppressor miR-375 in tongue cancer. J. Cell. Biochem. 116: 836–843, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: microRNA; miR-375; ANTI-CANCER DRUGS; TONGUE CANCER

Head and neck cancer is one of the most common cancers worldwide, and more than 90% of these cancers are squamous cell carcinomas [Siegel et al., 2012]. Among the head and neck cancers, oral cancer is the most common, with the tongue being one of the primary sites for this malignancy [Jemal et al., 2004]. The current treatment options for head and neck cancers include surgery, radiotherapy, and chemotherapy. Patients who are diagnosed with early-stage head and neck cancers (stages I and II) are treated with surgery, often combined with radiotherapy; the 5-year survival rate of this population ranges from 70–90%. On the other hand, the 5-year survival rate drops down to less than 50% for patients with advanced stage tumors, which limits the options for therapy [Argiris et al., 2008; Scully and Bagan, 2008]. Despite the fact that chemotherapy became a common option for cancer treatment a few decades ago, dose-limiting toxicity of the chemotherapeutic agents prevents their widespread clinical use [Le Tourneau Siu, 2008; Harari et al., 2009; Matta and Ralhan, 2009]. Therefore, there is a strong need to understand the biological and molecular actions of the current chemotherapeutic drugs in order to develop more effective treatment options that reduce toxicity and improve efficacy.

MicroRNA (miRNA) is a class of short endogenous non-coding RNA that contribute to posttranscriptional regulation of protein-coding genes in various biological processes [Ambros, 2004]. Recent studies have identified many deregulated miRNAs in human cancers that either accelerate the development of cancer or suppress its processes [Lee and Dutta, 2009; White et al., 2011]. The balance of these miRNAs needs to be stable in order to maintain homeostasis of the cells. However, when this balance is disrupted, cells behave abnormally; accumulation of abnormal cells can initiate cancer. The functions of these miRNAs in these diseases still remain unclear, and cancer researchers are only starting to understand their relevance in cancers.

Regarding the recent advances in miRNA research, it is important to understand the significance of these miRNAs in maintaining homeostasis and how their deregulation is involved in the

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development of tongue cancer. Moreover, understanding how miRNAs respond to therapeutics would provide insights into the development of future treatments for tongue cancer patients. Since miRNAs can simultaneously regulate multiple targets, it is important to investigate the effect of anti-cancer chemotherapeutic drugs on specific miRNAs.

In our previous study, we used human oral tumor samples to analyze altered miRNA expressions compared to controls [Jung et al., 2012]. The most significantly underexpressed miRNA was miR-375, and overexpressing miR-375 in CAL 27 tongue cancer cells inhibited common cancerous properties such as cell proliferation, migration, and invasion [Jung et al., 2013]. Moreover, miR-375 function as a negative regulator for multiple oncogenic genes including CIP2A, MYC, 14–3-3ζ, E6AP, and E6 and E7 viral genes produced from HPV-16 and -18 [Jung et al., 2014]. As a result, miR-375-mediated suppression of these molecules rescued downstream molecules such as p21, p53, and RB, indicating that the presence of miR-375 is important for tumor suppression. Consistent with the results observed in oral cancer, series of evidence indicate that miR-375 is a tumor suppressor in several other cancer types as well [Ding et al., 2010; Tsukamoto et al., 2010; Nohata et al., 2011; Wang et al., 2011; Harris et al., 2012; He et al., 2012; Kong et al., 2012]. Therefore, the main purpose of the current study was to test whether common anti-cancer drugs would reactivate miR-375. Here, we show reactivation of miR-375 by doxorubicin, 5-fluorouracil, trichostatin A, or etoposide treatment in tongue cancer cells.

MATERIALS AND METHODS

CELL LINES

CAL 27 and SCC-25 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). CAL 27 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1.5 g/L sodium bicarbonate; SCC-25 in DMEM/F12 supplemented with 15 mM HEPES, 1.2 g/L sodium bicarbonate, 0.5 mM sodium pyruvate, and 400 ng/ml hydrocortisone. Both media were supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin. Both cell lines were incubated in a humidified atmosphere of 37 °C with 5% CO_2.

DRUG TREATMENT

Cells were treated with doxorubicin (Fisher Scientific, BP2516–1), (Sigma-Aldrich, F6627), trichostatin A (Sigma-Aldrich, T8552), or etoposide (Sigma-Aldrich, E1383). Doxorubicin (Fisher Scientific, BP2516–1) was solubilized in nuclease-, protease-free molecular grade water. 5-fluorouracil (Sigma-Aldrich, F6627), trichostatin A (Sigma-Aldrich, T8552), and etoposide (Sigma-Aldrich, E1383) were solubilized in dimethyl sulfoxide (DMSO). The concentrations of the drugs used in this study were determined based on the preliminary data testing different dosages of the drugs on each cell line. Two 5-fold different concentrations indicated in the figures were selected to examine the effect on miR-375 expression change.

RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

RNA from the cells incubated with the anti-cancer reagents was isolated using the mirVana miRNA Isolation kit (Life Technologies). The quantity of RNA was measured by a NanoDrop ND-100 spectrophotometer (Nanodrop Technology Inc., Wilmington, DE). TaqMan MicroRNA Reverse Transcription kit or TaqMan High-Capacity cDNA Reverse Transcription kit (Life Technologies) were used to reverse transcribe corresponding cDNA for miRNA and mRNA, respectively. TaqMan Fast Universal PCR Master Mix (Life Technologies) was used for qPCR reactions and an Applied Biosystems StepOnePlus Real-Time PCR machine was used to measure the relative expression of miRNAs and mRNAs. Fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method [Pfaffl, 2001]. RNU44 was used to normalize miR-375 levels and 18 S rRNA was used as an internal control for mRNA and pri-miR-375 expression.

PATIENT SAMPLES

The human oral samples previously described (GSE28100) were used in the present study, and clinicopathological information is available from our previous report [Jung et al., 2012]. Fifteen tongue tumors were selected to analyze the expression of pri-miR-375. The TNM (tumor size, regional nodal involvement, presence or absence of distant metastasis) classification system determined the tumor stages. The tongue tumors were separated into two groups: early ($n = 6$) and advanced stage ($n = 11$). The human tissue samples were collected from the Tissue Bank of the Moffitt Cancer Center (Protocol #MCC-15370) and approved by the Institutional Review Board of the University of South Florida (# 106444). Written consent was given by the patients for their information to be stored in the Moffitt Tissue Bank and used for research.

BIOINFORMATICS

Four microarray data sets were retrieved from NCBI Gene Expression Omnibus. The mRNAs significantly ($p < 0.05$) different between cancer and normal samples that were mappable in Ingenuity Pathway Analysis (IPA) software (Qiagen) were selected as candidate targets. Using the microRNA filter from IPA software, the putative targets and known targets of miR-375 were subjected to further analysis.

STATISTICAL ANALYSES

The results from at least three independent experiments were expressed as mean ± S.D. Statistical analysis of the data was performed by student's *t* test or one-way analysis of variance, and the data from patient samples were analyzed by Mann-Whitney *U* test using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

RESULTS

DOXORUBICIN, 5-FLUOROURACIL, TRICHOSTATIN A, AND ETOPOSIDE INDUCE THE EXPRESSION OF MIR-375 IN CAL 27 AND SCC-25

Since miR-375 is underexpressed in oral cancers, we used two tongue cancer cell lines for this study, CAL 27 and SCC-25, to test the effect of anti-cancer therapeutic drugs on tumor suppressor miR-375. The response of miR-375 by the drugs was tested in two different concentrations, which varied for each drug. After 24 h

treatment of doxorubicin, 5-fluorouracil, trichostatin A, or etoposide, significant increase of miR-375 was detected in both cell lines. The increase was greater when the cells were treated with the higher concentration of drugs (Fig. 1). Although the increase of miR-375 varied depending on the drugs and the dosages, treatment with the drugs led to a 2- to 16-fold increase in miR-375 levels in CAL 27 (Fig. 1A), and a 2- to 12-fold increase in SCC-25 (Fig. 1B). This increase is more likely due to activation of miR-375 itself rather than activating overall miRNA biogenesis because miR-16 (experimentally shown control miRNA) expression remains unchanged in both cells (Fig. 1C and 1D). The selected "high" and "low" concentrations were based on preliminary experiments where CAL 27 was treated with doxorubicin at various doses as shown similarly in Figure 2. Based on this result, we hypothesized that miR-375 level increased when cells were treated with a drug dose high enough to decrease cell viability. Therefore, for other drugs, a high dose that significantly reduced cell viability of CAL 27 or SCC-25 was selected, and a 5-fold lower dose was chosen for miR-375 expression comparison.

DOXORUBICIN INCREASES MIR-375 LEVEL IN CAL 27 CELLS IN A DOSE-DEPENDENT MANNER

In order to examine whether miR-375 expression respond to anti-cancer drugs, we selected doxorubicin to test in CAL 27 cells as an example how miR-375 reactivation occurred linearly in a wider range of dose and time upon drug treatment (Fig. 2). The increase of miR-375 via doxorubicin was tested in CAL 27 using various concentrations of the drug. Cells were incubated for 24 h in medium containing 8 different concentrations of doxorubicin (2-fold serial dilutions from 0.003 to 1.7 μM). The level of miR-375 was elevated in a dose-dependent manner, indicating that miR-375 levels sensitively respond to doxorubicin treatment in these tongue cancer cells (Fig. 2A). The increase of miR-375 was inversely correlated with the number of viable cells (Fig. 2B), and reduction of the known miR-375 targets such as PDK1 [Tsukamoto et al., 2010], 14-3-3ζ [Tsukamoto et al., 2010], CIP2A [Jung et al., 2013], and E6AP [Jung et al., 2014] correlated with the increase of miR-375 in a dose-dependent manner (Fig. 2C); providing evidence that doxorubicin treatment reactivated the tumor suppressor function of miR-375 and did not simply increase transcript levels. These results suggest that the increase of miR-375 by doxorubicin is dose-dependent and functionally relevant in these tongue cancer cells.

DOXORUBICIN INCREASES MIR-375 LEVEL IN CAL 27 CELLS IN A TIME-DEPENDENT MANNER

In order to examine the kinetics of miR-375 expression during the doxorubicin treatment, CAL 27 cells were exposed to 1.7 μM doxorubicin for various amounts of time. An increase in miR-375

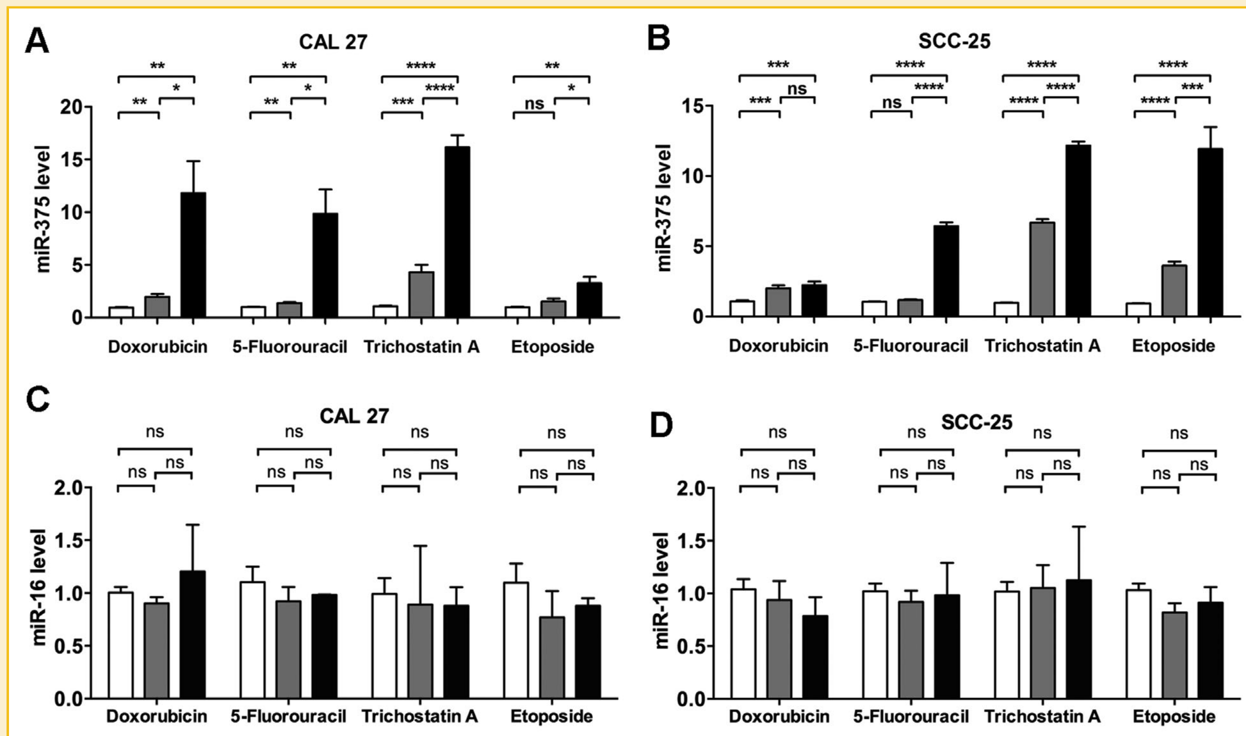


Fig. 1. Doxorubicin, 5-fluorouracil, trichostatin A, and etoposide induce the expression of miR-375 in CAL 27 and SCC-25. The level of miR-375 was measured in (A) CAL 27 and (B) SCC-25 cells 24 h after drug treatment, each with two different concentrations (5-fold difference between low and high concentrations). The level of miR-16 was measured as a negative control in (C) CAL 27 and (D) SCC-25 cells. CAL 27 and SCC-25 cells were treated with doxorubicin (0.34 and 1.7 μM), 5-fluorouracil (0.2 and 1.0 mM for CAL 27; 1.0 and 5.0 mM for SCC-25), trichostatin A (0.2 and 1.0 μM), and etoposide (40 and 200 μM for CAL 27; 200 and 1000 μM for SCC-25). White, grey, and black bars are untreated controls, low concentration treatment, and high concentration treatment, respectively. Results are expressed as mean ± SD from at least three independent experiments for miR-375, and duplicates or triplicates for miR-16. ns, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

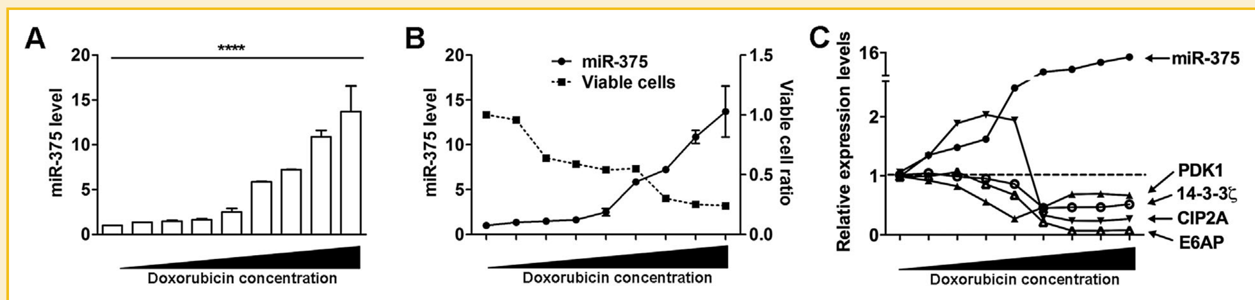


Fig. 2. Doxorubicin increases miR-375 level in CAL 27 cells in a dose-dependent manner. (A) CAL 27 cells were treated with doxorubicin for 24 h using 8 different concentrations (2-fold dilutions from 0.003 to 1.7 μM). (B) Comparison of dose-dependent increase of miR-375 and decrease of viable cell ratio. (C) The expression of miR-375 and its known targets in the 8 different concentrations used in panel A. The levels of miR-375 and mRNAs were compared to untreated CAL 27 cells. ****$p < 0.0001$.

level was observed starting at 4 h and it continued to increase up to the 24 h time point (Fig. 3A). This increase of miR-375 over time also correlated with reduction of viable CAL 27 cells (Fig. 3B). Interestingly, the primary transcript of miR-375 (pri-miR-375) gradually increased during the 24 h time points indicating that doxorubicin activated the transcription of miR-375 (Fig. 3C).

Since pri-miR-375 expression was elevated by doxorubicin (Fig. 3C), the level of pri-miR-375 was measured after treating CAL 27 and SCC-25 cells with doxorubicin, 5-fluorouracil, trichostatin A, and etoposide using two different concentrations as described in Figure 1. Pri-miR-375 was significantly elevated in both CAL 27 (Fig. 4A) and SCC-25 (Fig. 4B). These results indicate that these anti-cancer drugs induced transcription of pri-miR-375 (Fig. 4) and thus increased the level of mature miR-375 (Fig. 1).

THE EXPRESSION OF PRI-MIR-375 IS REDUCED IN TONGUE TUMORS COMPARED TO NORMAL TONGUES

Reduced expression of miR-375 has been reported in a number of cancer types [Ding et al., 2010; Tsukamoto et al., 2010; Nohata et al., 2011; Wang et al., 2011; He et al., 2012; Harris et al., 2012; Kong et al., 2012]. In particular, we have shown that miR-375 is down-regulated in oral cancers compared to healthy individuals [Jung et al., 2013]. Therefore, it was reasonable to examine whether the expression of pri-miR-375 was reduced in human tongue tumors. In order to test this hypothesis, 15 tongue tumors and 5 normal tongue samples were used to measure pri-miR-375 expression. Compared to normal tongues, the tumors had about a 2-fold decrease of pri-miR-375 (Fig. 5A). Reduction of pri-miR-375 was more obvious when the tumors were analyzed separately depending on their stages. "Early stage" includes tumors in stage 1 and 2, and "advanced stage" includes tumors of stage 3 and 4. A more significant reduction of pri-miR-375 was observed in the advanced tumors compared to normal tongues (3-fold decrease, $p < 0.01$, Fig. 5B). Early stage tumors showed a trend of decreasing pri-miR-375 but the decreases were not significant reductions, possibly due to small sample size. Therefore, this data suggests that transcription of pri-miR-375 is inhibited in advanced stage human tongue cancer patients, with potential inhibition shown in early stage tumors.

BIOINFORMATIC ANALYSES OF THE FUNCTIONS OF MIR-375 IN ORAL SQUAMOUS CELL CARCINOMAS

Since the significance of miR-375 as a tumor suppressor has been illustrated in tongue cancers, it is important to understand the candidate targets of miR-375 and to determine the protein-coding genes that are potentially misregulated by the reduction of miR-375 in this disease. Four sets of gene microarrays comparing oral tumors to normal oral tissues (GSE9844, GSE3524, GSE13601, and GSE31056) were analyzed by Ingenuity Pathway Analysis (IPA)

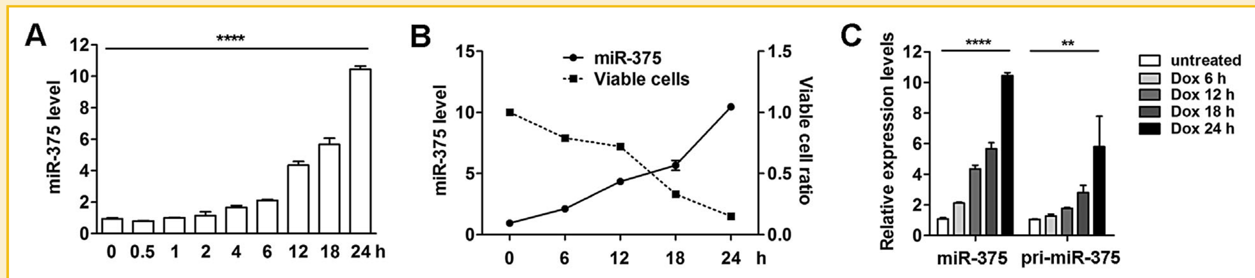


Fig. 3. Doxorubicin increases miR-375 level in CAL 27 cells in a time-dependent manner. (A) CAL 27 cells were treated with 1.7 μM of doxorubicin and the level of miR-375 was examined at 8 different time points (0.5 to 24 h). (B) Correlation of time-dependent increase of miR-375 and the decrease of viable cell ratio. (C) Comparison of miR-375 and pri-miR-375 expression in CAL 27 cells treated with 1.7 μM of doxorubicin at 4 different time points (6 to 24 h). The levels of miR-375 were compared to untreated CAL 27 cells. **$p < 0.01$ and ****$p < 0.0001$.

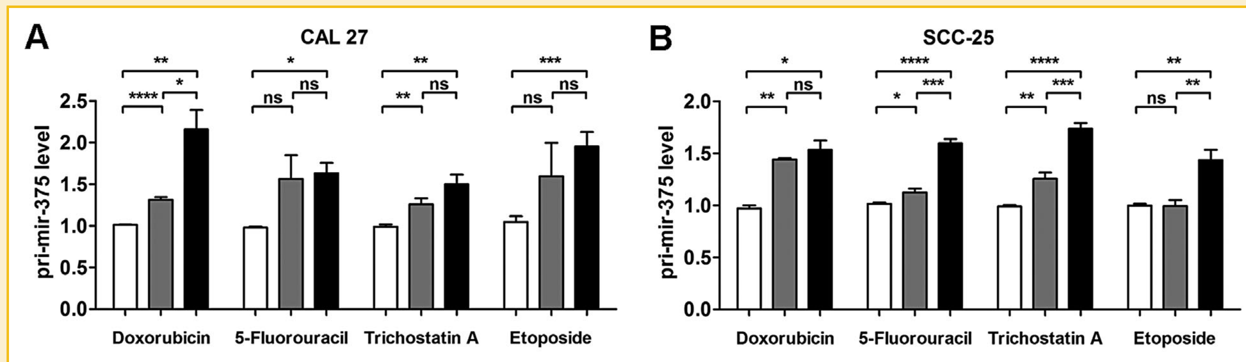


Fig. 4. Doxorubicin, 5-fluorouracil, trichostatin A, and etoposide induce the transcription of pri-miR-375 in a dose-dependent manner. The level of pri-miR-375 was measured in (A) CAL 27 and (B) SCC-25 cells 24 h after drug treatment with two different concentrations (5-fold difference between low and high concentrations). CAL 27 and SCC-25 cells were treated with doxorubicin (0.34 and 1.7 μM), 5-fluorouracil (0.2 and 1.0 mM for CAL 27; 1.0 and 5.0 mM for SCC-25), trichostatin A (0.2 and 1.0 μM), or etoposide (40 and 200 μM for CAL 27; 200 and 1000 μM for SCC-25). White, grey, and black bars are untreated controls, low concentration treatment, and high concentration treatment, respectively. Results are expressed as mean ± SD from at least three independent experiments. ns, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

software using a microRNA filter to select the genes that were overexpressed in the profiles. This analysis identified 65 genes as putative miR-375 targets. In addition to the 65 candidate genes, 23 known miR-375 targets were added to the list for further bioinformatic analyses (Fig. 6A). From the Gene Expression Omnibus (GEO) database, the four sets were selected based on the criteria that they were comparing oral squamous cell carcinoma, which is the most common type of oral cancer. In addition, profiles using similar microarray platforms were selected in order to minimize differences among assay methods (Fig. 6B). IPA clearly showed that the majority of the putative targets of miR-375 are associated with cancer through their involvement in regulation of cell death and survival, cell growth, proliferation, gene expression, and cell cycle (Fig. 6C). It is also important to note that these putative miR-375 targets form networks, indicating possible associations with and regulation of each other (Fig. 6D). Therefore, overexpression of these potential targets of miR-375 via silencing of miR-375 in tongue cells may play a larger role than expected in coordinating important functions in tongue cancer development.

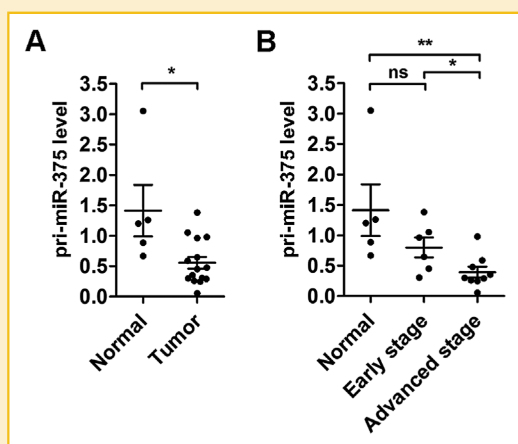
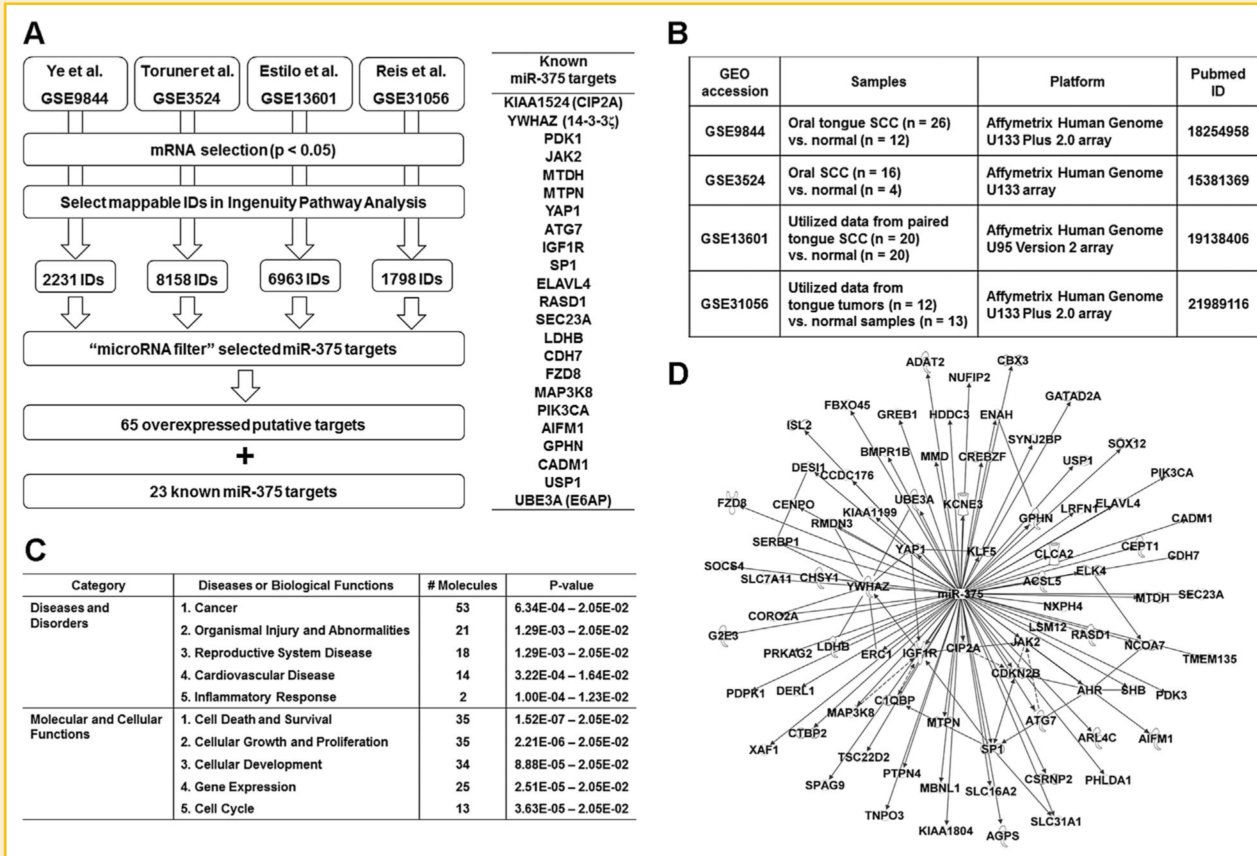


Fig. 5. The expression of pri-miR-375 is reduced in tongue tumors compared to normal tongues. (A) The pri-miR-375 transcript levels were compared between normal tongues ($n = 5$) and tongue tumors ($n = 15$) using qRT-PCR. (B) The pri-miR-375 transcript levels were then compared among normal tongues ($n = 5$), early stage tongue tumors ($n = 6$), and advanced stage tongue tumors ($n = 9$). ns, not significant, **$p < 0.01$.

DISCUSSION

Chemotherapy is one of the key treatments for oral cancers. Decisions are made whether or not to combine chemotherapeutic treatment with radiotherapy and/or surgery depending on the condition of the tumor, and it is common to use two or three different types of chemotherapeutic agents to interrupt different stages within the life cycles of cancers [Furness et al., 2011]. Since anti-cancer drugs are a common option for therapy, it is important to understand how cancer cells respond to the current chemotherapeutic drugs in order to design and produce treatment options with minimal side effects. In this study, we demonstrate the response of miR-375 in tongue cancer to the common anti-cancer therapeutic drugs doxorubicin, 5-fluoruracil, trichostatin A, and etoposide. Increase of miR-375 in two tongue cancer cell lines (CAL 27 and SCC-25) upon treatment with these drugs shows that miR-375 may mediate cellular reactions in response to these drugs.

Evidence shows that these anti-cancer drugs alter miRNA expression in a cell-type specific manner. For example, gastric cancer cells exposed to trichostatin A show increased miR-375 levels [Tsukamoto et al., 2010]. In another study, a low concentration (50 nM) of doxorubicin was used for 2–5 days to trigger cellular senescence and increase miR-375 levels in erythroleukemic cells [Yang et al., 2012]. Other anti-cancer drugs such as paclitaxel (mitotic inhibitor), 4-phenylbutyric acid (histone deacetylase inhibitor), or 5-aza-2′deoxycytidine (DNA methyltransferase inhibitor) could activate miR-375 expression in different cell types, indicating a potential that anti-cancer drugs may activate miR-375 via diverse mechanisms [Tsukamoto et al., 2010; Mazar et al., 2011;



GEO accession	Samples	Platform	Pubmed ID
GSE9844	Oral tongue SCC (n = 26) vs. normal (n = 12)	Affymetrix Human Genome U133 Plus 2.0 array	18254958
GSE3524	Oral SCC (n = 16) vs. normal (n = 4)	Affymetrix Human Genome U133 array	15381369
GSE13601	Utilized data from paired tongue SCC (n = 20) vs. normal (n = 20)	Affymetrix Human Genome U95 Version 2 array	19138406
GSE31056	Utilized data from tongue tumors (n = 12) vs. normal samples (n = 13)	Affymetrix Human Genome U133 Plus 2.0 array	21989116

Category	Diseases or Biological Functions	# Molecules	P-value
Diseases and Disorders	1. Cancer	53	6.34E-04 – 2.05E-02
	2. Organismal Injury and Abnormalities	21	1.29E-03 – 2.05E-02
	3. Reproductive System Disease	18	1.29E-03 – 2.05E-02
	4. Cardiovascular Disease	14	3.22E-04 – 1.64E-02
	5. Inflammatory Response	2	1.00E-04 – 1.23E-02
Molecular and Cellular Functions	1. Cell Death and Survival	35	1.52E-07 – 2.05E-02
	2. Cellular Growth and Proliferation	35	2.21E-06 – 2.05E-02
	3. Cellular Development	34	8.88E-05 – 2.05E-02
	4. Gene Expression	25	2.51E-05 – 2.05E-02
	5. Cell Cycle	13	3.63E-05 – 2.05E-02

Fig. 6. Bioinformatic analyses of the functions of miR-375 in oral squamous cell carcinomas. (A) Four microarray data sets from Gene Expression Omnibus (GEO) were used to select mRNAs that are differentially expressed between tongue cancer and normal specimens. Ingenuity Pathway Analysis (IPA) microRNA filter identified 65 putative miR-375 targets that are overexpressed in tongue cancer. Twenty-three known miR-375 targets added in the analysis are listed. (B) Information regarding the analyzed microarray data sets. (C) List of top 5 significant disease and biological functions related to miR-375 targets. (D) Molecular network analysis of miR-375 targets using IPA.

Shen et al., 2013]. While we did not see activation of miR-16 in CAL 27 and SCC-25 upon treatment with these anti-cancer drugs, others reported that etoposide and 5-fluorouracil [Wang et al., 2013] could increase the expression of miR-16 in gastric cancer cells, and doxorubicin could induce miR-16 in a p53-dependent manner [Lezina et al., 2013]. These results from different cell types suggest a cell-type variation of drug-responsive mechanisms for regulating miRNAs. Together, these reports indicate that the miRNA responses to these drug treatments, either activation or suppression, are variable depending on the cell type and the upstream molecules responsible for changes of each miRNA.

Gradual increase of both miR-375 and pri-miR-375 in time-dependent doxorubicin treatments is consistent with the notion that transcription of pri-miR-375 was stimulated by the drug treatments. Importantly, the lower level of pri-miR-375 in human tongue tumors compared to the normal tongues explains, in part, the reduction of miR-375 in this disease. Therefore, identifying functional miR-375 targets is important for understanding the pathology of tongue cancer. Utilizing the benefit of the publicly available gene expression data sets of oral cancer, the overexpressed genes that are predicted as miR-375 targets were selected for further analysis. According to our bioinformatics analysis shown in Figure 6, these candidate targets are heavily involved in regulating biological functions that are necessary for cancer. These results emphasize the importance of miR-375 in oral cancers. Since miR-375 activation was a common phenomenon produced by all the drugs tested in this study, it is possible that these targets are subjected to miR-375-mediated suppression upon drug treatment, and may partially explain the overall tumor suppressive mechanism of the drugs. Further investigation is required to fully understand the functional influence of drug-induced miR-375 on its targets and the potential direct effects of the drugs on these targets.

The initial studies on miR-375 have focused on its function in regulating glucose-induced insulin secretion in pancreatic cells [Poy et al., 2004]. miR-375 plays an important role in cell proliferation and maintains pancreatic β-cell mass during development as demonstrated in mice and zebrafish with engineered genetic deletion of miR-375 [Kloosterman et al., 2007; Poy et al., 2009]. The connection between reduced miR-375 levels and increased proliferation has been emphasized in different types of cancers. For example, similar to our observation of underexpressed miR-375 in oral cancer [Jung et al., 2013], reduced expression and the tumor suppressive function of miR-375 have been shown in cervical cancer [Wang et al., 2011], head and neck cancer [Nohata et al., 2011; Harris

et al., 2012], hepatocellular cancer [He et al., 2012], esophageal cancer [Kong et al., 2012], and gastric cancer [Ding et al., 2010; Tsukamoto et al., 2010]. These studies suggest that dysregulation of miR-375 may play an important role in diverse types of cancer. While underexpression of miR-375 has been reported in a number of cancers, the mechanism of the deregulation is still unclear. One of the known mechanisms for miR-375 regulation in oral cells is via DNA methylation. DNA methylation status has been tested in sets of miRNAs that inhibited oral squamous cell carcinoma proliferation [Uesugi et al., 2011]. As shown in their supplementary data, miR-375 was found to be one of the miRNAs that were hypermethylated in more than 50% of OSCC lines examined. Since epigenetic regulation of gene expression is involved in cell fate, it is also important to understand the dynamics of miRNAs and their response to chemotherapies. Elucidation of cellular mechanistic responses to these drugs would aid in comprehending miR-375 deregulation in cancer. This includes, but is not limited to, epigenetic deregulation, mutation, transcriptional deregulation, and DNA copy number abnormalities [Deng et al., 2008]. Our findings suggest a possibility that chemotherapeutic drugs act on tongue cancer cells to prevent cancer growth partially via changing the transcription status of miR-375; a more sophisticated investigation is needed to determine how the epigenetic events involving miR-375 happen in tongue cancer.

Understanding the functional association between loss or gain of particular miRNAs and cancerous phenotypes will provide significant insight for diagnostics, prognostics, and therapeutics for oral cancer. Such information may not be limited to developing cures for oral cancer but may also apply to other cancers, as well as broader applications to other diseases. Further mechanistic studies on the relationship between the action of anti-cancer drugs and miRNA may provide valuable insights for developing better strategies in tongue cancer treatment.

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REFERENCES

Ambros V. 2004. The functions of animal microRNAs. Nature 431:350–355.

Argiris A, Karamouzis MV, Raben D, Ferris RL. 2008. Head and neck cancer. Lancet 371:1695–1709.

Deng S, Calin GA, Croce CM, Coukos G, Zhang L. 2008. Mechanisms of microRNA deregulation in human cancer. Cell Cycle 7:2643–2646.

Ding L, Xu Y, Zhang W, Deng Y, Si M, Du Y, Yao H, Liu X, Ke Y, Si J, Zhou T. 2010. MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. Cell Res 20:784–793.

Furness S, Glenny AM, Worthington HV, Pavitt S, Oliver R, Clarkson JE, Macluskey M, Chan KK, Conway DI. 2011. Interventions for the treatment of oral cavity and oropharyngeal cancer: chemotherapy. Cochrane Database Syst Rev. CD006386.

Harari PM, Wheeler DL, Grandis JR. 2009. Molecular target approaches in head and neck cancer: epidermal growth factor receptor and beyond. Semin Radiat Oncol 19:63–68.

Harris T, Jimenez L, Kawachi N, Fan JB, Chen J, Belbin T, Ramnauth A, Loudig O, Keller CE, Smith R, Prystowsky MB, Schlecht NF, Segall JE, Childs G. 2012. Low-level expression of miR-375 correlates with poor outcome and metastasis while altering the invasive properties of head and neck squamous cell carcinomas. Am J Pathol 180:917–928.

He XX, Chang Y, Meng FY, Wang MY, Xie QH, Tang F, Li PY, Song YH, Lin JS. 2012. MicroRNA-375 targets AEG-1 in hepatocellular carcinoma and suppresses liver cancer cell growth in vitro and in vivo. Oncogene 31:3357–3369.

Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ, American Cancer S. 2004. Cancer statistics, 2004. CA Cancer J Clin 54:8–29.

Jung HM, Patel RS, Phillips BL, Wang H, Cohen DM, Reinhold WC, Chang LJ, Yang LJ, Chan EKL. 2013. Tumor suppressor miR-375 regulates MYC expression via repression of CIP2A coding sequence through multiple miRNA-mRNA interactions. Mol Biol Cell 24:1638–1648.

Jung HM, Phillips BL, Chan EKL. 2014. MiR-375 activates p21 and suppresses telomerase activity by coordinately regulating HPV E6/E7, E6AP, CIP2A, and 14-3-3zeta. Mol Cancer 13:80.

Jung HM, Phillips BL, Patel RS, Cohen DM, Jakymiw A, Kong WW, Cheng JQ, Chan EKL. 2012. Keratinization-associated miR-7 and miR-21 regulate tumor suppressor reversion-inducing cysteine-rich protein with kazal motifs (RECK) in oral cancer. J Biol Chem 287:29261–29272.

Kloosterman WP, Lagendijk AK, Ketting RF, Moulton JD, Plasterk RH. 2007. Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. PLoS Biol 5:e203.

Kong KL, Kwong DL, Chan TH, Law SY, Chen L, Li Y, Qin YR, Guan XY. 2012. MicroRNA-375 inhibits tumour growth and metastasis in oesophageal squamous cell carcinoma through repressing insulin-like growth factor 1 receptor. Gut 61:33–42.

Le Tourneau C, Siu LL. 2008. Molecular-targeted therapies in the treatment of squamous cell carcinomas of the head and neck. Curr Opin Oncol 20:256–263.

Lee YS, Dutta A. 2009. MicroRNAs in cancer. Annu Rev Pathol 4:199–227.

Lezina L, Purmessur N, Antonov AV, Ivanova T, Karpova E, Krishan K, Ivan M, Aksenova V, Tentler D, Garabadgiu AV, Melino G, Barlev NA. 2013. MiR-16 and miR-26a target checkpoint kinases Wee1 and Chk1 in response to p53 activation by genotoxic stress. Cell Death Dis 4:e953.

Matta A, Ralhan R. 2009. Overview of current and future biologically based targeted therapies in head and neck squamous cell carcinoma. Head Neck Oncol 1:6.

Mazar J, DeBlasio D, Govindarajan SS, Zhang S, Perera RJ. 2011. Epigenetic regulation of microRNA-375 and its role in melanoma development in humans. FEBS Lett 585:2467–2476.

Nohata N, Hanazawa T, Kikkawa N, Mutallip M, Sakurai D, Fujimura L, Kawakami K, Chiyomaru T, Yoshino H, Enokida H, Nakagawa M, Okamoto Y, Seki N. 2011. Tumor suppressive microRNA-375 regulates oncogene AEG-1/MTDH in head and neck squamous cell carcinoma (HNSCC). J Hum Genet 56:595–601.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M. 2004. A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432:226–230.

Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M, Stoffel M. 2009. MiR-375 maintains normal pancreatic alpha- and beta-cell mass. Proc Natl Acad Sci U S A 106:5813–5818.

Sabirzhanov B, Zhao Z, Stoica BA, Loane DJ, Wu J, Borroto C, Dorsey SG, Faden AI. 2014. Downregulation of miR-23a and miR-27a following experimental traumatic brain injury induces neuronal cell death through activation of proapoptotic Bcl-2 proteins. J Neurosci 34:10055–10071.

Scully C, Bagan JV. 2008. Recent advances in Oral Oncology 2007: imaging, treatment and treatment outcomes. Oral Oncol 44:211–215.

Shen Y, Wang P, Li Y, Ye F, Wang F, Wan X, Cheng X, Lu W, Xie X. 2013. MiR-375 is upregulated in acquired paclitaxel resistance in cervical cancer. Br J Cancer 109:92–99.

Siegel R, Naishadham D, Jemal A. 2012. Cancer statistics, 2012. CA Cancer J Clin 62:10–29.

Tsukamoto Y, Nakada C, Noguchi T, Tanigawa M, Nguyen LT, Uchida T, Hijiya N, Matsuura K, Fujioka T, Seto M, Moriyama M. 2010. MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. Cancer Res 70:2339–2349.

Uesugi A, Kozaki K, Tsuruta T, Furuta M, Morita K, Imoto I, Omura K, Inazawa J. 2011. The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. Cancer Res 71:5765–5778.

Wang F, Li Y, Zhou J, Xu J, Peng C, Ye F, Shen Y, Lu W, Wan X, Xie X. 2011. MiR-375 is down-regulated in squamous cervical cancer and inhibits cell migration and invasion via targeting transcription factor SP1. Am J Pathol 179:2580–2588.

Wang F, Song X, Li X, Xin J, Wang S, Yang W, Wang J, Wu K, Chen X, Liang J, Tian J, Cao F. 2013. Noninvasive visualization of microRNA-16 in the chemoresistance of gastric cancer using a dual reporter gene imaging system. PLoS One 8:e61792.

White NM, Fatoohi E, Metias M, Jung K, Stephan C, Yousef GM. 2011. Metastamirs: a stepping stone towards improved cancer management. Nat Rev Clin Oncol 8:75–84.

Yang MY, Lin PM, Liu YC, Hsiao HH, Yang WC, Hsu JF, Hsu CM, Lin SF. 2012. Induction of cellular senescence by doxorubicin is associated with upregulated miR-375 and induction of autophagy in K562 cells. PLoS One 7:e37205.