Keratinization-associated miR-7 and miR-21 Regulate Tumor Suppressor Reversion-inducing Cysteine-rich Protein with Kazal Motifs (RECK) in Oral Cancer

Background: The role of miRNA-mediated regulation of RECK in keratinized tumors is unclear.

Results: miRNAs express differentially in subtypes of OSCCs, and keratinization-associated miRNAs inversely correlate with RECK in oral cancer cells.

Conclusion: miR-7 and miR-21 negatively regulate the tumor suppressor gene RECK.

Significance: Keratinization-associated miRNAs may serve as novel targets to reduce tumor aggressiveness.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression during many biological processes. Recently, the aberrant expressions of miRNAs have become a major focus in cancer research. The purpose of this study was to identify deregulated miRNAs in oral cancer and further focus on specific miRNAs that were related to patient survival. Here, we report that miRNA expression profiling provided more precise information when oral squamous cell carcinomas were subcategorized on the basis of clinicopathological parameters (tumor primary site, histological subtype, tumor stage, and HPV16 status). An innovative radar chart analysis method was developed to depict subcategories of cancers taking into consideration the expression patterns of multiple miRNAs combined with the clinicopathological parameters. Keratinization of tumors and the high expression of miR-21 were the major factors related to the poor prognosis of patients. Interestingly, a majority of the keratinized tumors expressed high levels of miR-21. Further investigations demonstrated the regulation of the tumor suppressor gene reversion-inducing cysteine-rich protein with kazal motifs (RECK) by two keratinization-associated miRNAs, miR-7 and miR-21. Transfection of miR-7 and miR-21-mimics reduced the expression of RECK through direct miRNA-mediated regulation, and these miRNAs were inversely correlated with RECK in CAL 27 orthotopic xenograft tumors. Furthermore, a similar inverse correlation was demonstrated in CAL 27 cells treated in vitro by different external stimuli such as trypsinization, cell density, and serum concentration. Taken together, our data show that keratinization is associated with poor prognosis of oral cancer patients and keratinization-associated miRNAs mediate deregulation of RECK which may contribute to the aggressiveness of tumors.

Oral cancer is one of the most prevalent cancers worldwide, with squamous cell carcinomas being the most common type, accounting for ~90% of all oral cancers (1). Approximately 300,000 new cases of oral cancers (amounting to 3% of total cancers) are anticipated annually (2). Although many studies have demonstrated how clinical and histological staging may explain why some cancers, but not others, behave aggressively, these stagings do not always properly reflect the extent of disease (3). A better prediction for the prognosis of patients has been proposed using gene expression data in combination with OSCC tumor stage information rather than tumor stage information alone (4, 5). Despite the medical advances and new treatments for oral cancer, the average five-year survival rate of 50% has not improved for decades (6). Hence, more molecular insights into oral cancer pathogenesis are needed to develop proper diagnostics and therapeutics.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs, ~18–25 nucleotides in length, that act as posttranscriptional regulators of gene expression in diverse cellular processes such as proliferation, differentiation, development, and cell death (7). Over 60% of all mammalian miRNAs are predicted targets of miRNAs, indicating their extensive roles in the regulation of numerous cellular processes (8). The discovery of miRNAs and the elucidation of their function in regulating
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gene expression levels demonstrates their increasing importance in cancer genetics (9). In cancer, some miRNAs have been found to play roles as regulators for tumor suppressors or oncogenes, depending on the tumor microenvironment and/or tissue type.

When epithelial cells keratinize, keratins and keratin filament-associated proteins are synthesized and assembled to form keratin filaments (10). During these processes, signaling molecules such as matrix metalloproteinases (MMPs) modulate the process of keratinization (10, 11). In particular, MMP-9 has been implicated in the process of keratinization (11). MMPs are well known to play crucial roles for tumor progression by degrading the extracellular matrix barriers encompassing the tumor and permitting invasion into surrounding connective tissues (12). At least three cancer-associated MMPs (MMP-2, MMP-9, and MTI-MMP) are regulated by RECK (13, 14). Reversion-inducing cysteine-rich protein with kazal motifs (RECK) is a membrane-anchored glycoprotein detected in a variety of normal human tissues (14). The reduced expression of RECK has been observed in various types of tumor tissues and is frequently associated with poor prognosis (15–19). Therefore, studies of the relation between RECK and keratinization may yield important insights into the mechanisms of carcinogenesis and the prognosis of oral cancer patients.

Although the majority of oral cancers are OSCCs, the heterogeneity within OSCCs and the limited molecular data regarding carcinogenesis hamper our understanding of the biological differences in OSCC subclasses. Therefore, the aim of this study was to identify prognostic indicators for oral cancer and to understand how aberrantly expressed miRNAs and their targets are involved in the pathogenesis of this disease.

EXPERIMENTAL PROCEDURES

Patients and Tissue Samples—All human OSCCs and normal tongue tissues were collected from the Tissue Bank at the Moffitt Cancer Center (Protocol no. MCC-15370) and approved by the Institutional Review Board of the University of South Florida (no. 106444). Written consent was given by the patients for their information to be stored in the Moffitt Tissue Bank and used for research. Clinicopathological characteristics of these 17 patients are shown in supplemental Table S1. Tissues were processed and analyzed using the Applied Biosystems Affymetrix Genotyping and annotation kit for miRNA and mRNA, respectively (Applied Biosystems). A NanoDrop ND-100 spectrophotometer (Nanodrop Technology, Inc., Wilmington, DE) was used to quantify the isolated RNA. Additionally, an Agilent 2100 bioanalyzer from the Interdisciplinary Center for Biotechnology Research at the University of Florida was used to monitor the size distribution and overall quality of total RNA prior to miRNA profiling.

MicroRNA Microarray Profiling—Total RNAs isolated from three normal tongues and 17 OSCCs were processed by Asuragen Services (Austin, TX) according to standard operating procedures. The Agilent human miRNA microarrays Rel1.2.0 (Agilent, Santa Clara, CA) according to the Agilent miRNA protocol v2.1. Cluster 3.0 (for dendrograms) (20) and Java TreeView (for heat map) (21) software programs were used to visualize the hierarchical clustering of miRNA expression in 17 OSCCs.

Quantitative Real-time PCR (qRT-PCR)—Reverse transcription was performed using the TaqMan MicroRNA reverse transcription kit or TaqMan high-capacity cDNA reverse transcription kit for miRNA and mRNA, respectively (Applied Biosystems, Foster City, CA). Quantification of miRNA and mRNA expression was performed using TaqMan MicroRNA assays, TaqMan gene expression assays, and TaqMan Fast Universal PCR Master Mix (Applied Biosystems). The experiments were processed and analyzed using the Applied Biosystems StepOne real-time PCR machine. Fold change values were calculated using the 2 ΔΔCt method. In addition to the three normal tissues used for the microarray analyses, two normal tongue tissues were added in the qRT-PCR analyses to enhance statistical computation. U6 snRNA and 18S rRNA were used as internal controls to normalize all the miRNA or mRNA expressions. TaqMan primer sets were used to measure mRNA levels of HPV16-E6 and HPV16-E7 using real-time PCR (22), which are more reliable assays compared with the oversensitive DNA PCR assays (22, 23). The degree of HPV status was determined by the corresponding cycle threshold (Ct) values: −, undetectable; +, Ct > 32; ++, 25 < Ct < 32; +++, Ct < 25.

Combinatorial Radar Chart Analyses—OSCCs were separated into subgroups according to their clinicopathological characteristics (supplemental Table S1). The tumors were separated on the basis of the primary site (oropharynx or oral cav-
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Differential miRNA Expression in OSCC Subtypes—Microarray expression profiling of 17 OSCCs compared with three normal tongue tissues identified significant (p < 0.05) differences in the expression levels of 134 miRNAs. In particular, a heat map using supervised hierarchical clustering analyses with criteria of p < 0.05 and a Log2 difference over 1.5 demonstrated that OSCCs had seven underexpressed and 62 overexpressed miRNAs when compared with normal tissues (Fig. 1A). Moreover, the tumors formed a hierarchical clustering of groups (a distinct cluster indicates the differential expression between normal (N1-N3, gray) and OSCC samples (other colors) depending on their clinicopathological parameters (Fig. 1A and supplemental Table S1). For example, six of seven oropharynx samples (brown) were clustered into a group in which five of the six were recorded as tumors originating at the base of the tongue. All of these tumors (T1284C, T3019C, T1715B, T661B, and T1231D) were non-keratinizing tumors (light purple). In addition, 10 HPV16-positive tumors formed two clusters: four (T3982D, T1373B, T4182B, and T577E) keratinizing and six (T426D, T1284C, T3019C, T1715B, T661B, and T1231D) non-keratinizing tumors all derived from oropharynx regions (Fig. 1A). Of the 69 aberrantly expressed miRNAs identified in the microarray analysis, nine up-regulated miRNAs (miR-9*, miR-424, miR-7–1*, miR-15b, miR-9, miR-21, miR-155, and miR-196a) and one down-regulated miRNA (miR-486-5p) were selected for further verification by Taqman qRT-PCR. Selection of miRNAs was performed on the basis of the fold changes and the p values from our microarray data, although some miRNAs were chosen because of their potential involvement in oncogenesis (e.g. miR-196a (28), miR-155 (29), and miR-21 (30–32)).

Combined Clinicopathological Parameters and miRNA Expression Signatures for OSCC Subtypes—Because the tumors formed distinct clusters on the basis of our microarray data
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(Fig. 1A), the results obtained from the qRT-PCR experiments were further analyzed for the differences among the subtypes of OSCCs instead of considering all 17 tumors as a single homogeneous group. Interestingly, significantly different miRNA expression levels were observed when subtypes of OSCCs were taken into consideration, as shown in Fig. 1, B–E. These observations indicated that the heterogeneous expression of miRNAs in OSCCs depends on their individual characteristics.
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Radar chart analyses were used to visualize the expression patterns of the 10 miRNAs selected for verification in individual OSCCs. When all 17 tumors were analyzed together, a complex heterogeneity was observed (supplemental Fig. S1A). In contrast, normal tissues exhibited similar contours, with the exception of one sample (supplemental Fig. S1B, N2). Interestingly, when using different combinations of these clinicopathological parameters, unique miRNA expression patterns were generated for specific subtypes of OSCCs (Fig. 2). Each subtype of tumor formed a contour that was easily distinguishable from that of the overall tumor population (Fig. 2 and supplemental Fig. S1A). Thus, these data demonstrated that combinatorial interpretations of clinicopathological features and miRNA expression profiles could be used as specific signatures of individual subtypes of oral tumors.

Advanced Tumor Stage, Keratinization State, and High Expression of miR-21 Are Indicators of Poor Prognosis for Oral Cancer Patients—The overall 5-year survival rate for the patients in this study was 41.2% (7 of 17, Supplemental Table S1). Clinicopathological parameters and miRNA expression patterns of OSCCs were examined to evaluate their correlation to the survival rate of the patients. Kaplan-Meier survival analyses demonstrated that patients with advanced stage tumors (5-year survival, 27.3% (3/11); hazard ratio, 3.36; 95% confidence interval, 1.08–10.49; \( p = 0.037 \)) and/or keratinizing tumors (5-year survival, 11.1% (1/9); hazard ratio, 3.34; 95% confidence interval, 1.10–10.12; \( p = 0.033 \)) were significantly associated with a poor survival prognosis (Fig. 3, A and B). From our clinical data, we found that 89% of patients (eight of nine) with keratinized tumors survived less than 3 years (36 months) compared with 75% of patients (six of eight) with non-keratinized tumors who survived more than 5 years (60 months) (supplemental Table S1). Primary site (\( p = 0.66 \)) and HPV status (\( p = 0.80 \)) were not associated with the prognosis of patients (supplemental Fig. S2A). Patients with tumors expressing high levels of miR-21, however, displayed a significant correlation (5-year survival, 12.5% (1/8); hazard ratio, 5.31; 95% confidence interval, 1.08–10.49; \( p = 0.037 \)) and/or keratinizing tumors (5-year survival, 11.1% (1/9); hazard ratio, 3.34; 95% confidence interval, 1.10–10.12; \( p = 0.033 \)) and/or keratinizing tumors (5-year survival, 11.1% (1/9); hazard ratio, 3.34; 95% confidence interval, 1.10–10.12; \( p = 0.033 \)) were significantly associated with a poor survival prognosis (Fig. 3, A and B).
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T1117X, T470A, and T1373B) of the eight tumors that expressed high levels of miR-21 were classified as keratinizing tumors. The only non-keratinizing tumor (T3982D) in the “high miR-21-expressing tumor” group had the lowest miR-21 expression of the eight tumors. Thus, these results suggested that the expression of miR-21 was associated with the keratinization of tumors and that this association could potentially contribute to the poorer survival of the patients.

**Coordinated Expression of Keratinization-associated miRNAs in Human Tissues and Cell Lines**

Our findings that both high miR-21 levels and the keratinization status were related to the survival rate of oral cancer patients made us pay particular attention to the expression levels of three miRNAs (miR-21, miR-7, and miR-424) that were found to be up-regulated in keratinizing tumors compared with either normal controls or non-keratinizing tumors (Fig. 1E). Using linear regression analysis, we observed a significant correlation when the expression levels of these miRNAs were examined together (Fig. 4). In human tissues (five normal and 17 OSCCs), miR-21 expression was directly correlated with both miR-7 and miR-424 expression levels (Fig. 4A). In human cell lines (seven head and neck cancer cells, four cervical cancer cells, and HEK293 cells), miR-21 and miR-7 expression directly correlated with miR-424 expression levels but not to each other (Fig. 4B). Together, these data suggested that the keratinization-associated miRNAs may have redundant regulatory effects for a common target.

**RECK as a Common Target of Keratinization-associated miRNAs**

—in silico analysis was performed to predict candidate targets coregulated by the three keratinization-associated miRNAs. Using several different prediction algorithms (supplemental Fig. S3), RECK was selected for further validation because RECK is a known tumor suppressor underexpressed in cancers. Putative binding sites for miR-7, miR-21, and miR-424 were detected, mostly on the 3’ UTR of RECK, with the exception of a second site for miR-7, which was also found within the coding region (Fig. 5A). The mRNA level of RECK was determined to be significantly underexpressed in the 17 OSCCs compared with the normal tissues (Fig. 5B). Next, the expression of RECK and miRNAs were analyzed by linear regression analysis, and an inverse correlation was observed between miR-7 and RECK in human tissues (Fig. 5C). When the same strategy was applied to human cancer cell lines, miR-21 and RECK were inversely correlated with each other (Fig. 5D). No correlation was observed, however, between miR-424 and RECK in either tissues or cell lines, indicating that miR-424 may not be a key regulator of RECK expression. Thus, these results showed that two keratinization-associated miRNAs, miR-7 and miR-21, could be important for regulating the expression of RECK.

A similar inverse correlation in the expression of miR-7, miR-21, and RECK were observed in CAL 27 orthotopic xenograft tumors. These were keratinizing tumors on the basis of H&E staining as confirmed by oral pathologists in the Depart-
ment of Oral and Maxillofacial Diagnostic Sciences and documented in our earlier study (26). In particular, the expression of miR-7 and miR-21 were increased in the CAL 27 xenograft tumors (Fig. 5, E and F, respectively) compared with normal human tongue tissues. Meanwhile, RECK expression was reduced in these tumors (Fig. 5G).

Direct Regulation of RECK by miR-7 and miR-21—To investigate the effects of miR-7 and miR-21 on the regulation of RECK in oral cancer cells, CAL 27 cells were transfected with either a miR-7-mimic, miR-21-mimic, or both. Although the transfection of miR-7- and miR-21-mimics successfully increased the level of each specific miRNA, the relative fold increase was different for each even though the same concentrations of miRNA-mimics were used (Fig. 6, A and B). RECK mRNA levels were measured 72 h post-transfection. Despite the different degree of increases in miRNA levels upon transfection, miR-21-mediated regulation showed a stronger repression (~ 30%) effect on RECK mRNA level compared with miR-
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7-mediated suppression (10–20%, Fig. 6C). Cotransfection of the two miRNA-mimics at the same final concentrations (each 12.5 nM used) suppressed RECK mRNA levels similar to the level shown by miR-21 single transfection (25 nM) (Fig. 6C). The regulation of RECK by miR-7 and miR-21 was not limited to CAL 27 cells. A similar reduction was observed in HeLa cells 48 h post-transfection with the miRNA-mimics (supplemental Fig. S4). RECK protein levels were monitored in HEK293 cells transfected with miRNA mimics. Strong repression (30–40%) of RECK protein was observed when transfected with miR-21-mimic alone, whereas miR-7 alone generated a mild repression (>10%, Fig. 6D). The data from HEK293 cells were presented because the protein level of RECK was barely detectable in untransfected CAL 27 cells. The direct regulation of RECK by miR-7 and miR-21 was further evaluated by luciferase reporter assays. The wild-type RECK 3′ UTR containing both putative miRNA binding sites were cloned downstream of firefly luciferase reporter. Mutated versions of the RECK 3′ UTR were generated for the putative binding sites for miR-7 or miR-21. Each mutants contained four altered nucleotides on the miRNA seed region binding sequence on RECK (Fig. 6, E and F).

Inverse Correlation of RECK and Keratinization-associated miRNAs Depending on Serum Concentration, Cell Density, and Adherence of CAL 27 Cells—RECK is required at the cellular level for stable cell substrate adhesion, and its expression is affected by external physiological stimuli such as cell density and serum concentration (33, 34). Thus, we investigated the expression of RECK and the keratinization-associated miRNAs in CAL 27 cells seeded in varying conditions of nutrient supply and confluence. Different concentrations of FBS ranging from 20% to 0% inversely affected the expression of both RECK and miR-7 (Fig. 7A). RECK expression gradually increased when serum was increasingly deprived, whereas miR-7 expression decreased. In contrast, miR-21 expression levels remained constant despite different concentrations of FBS, suggesting that RECK was not regulated by miR-21 under these conditions.

Cell density was another factor that inversely affected the expression of RECK and miR-7 (Fig. 7B). CAL 27 cells were plated at different densities by 2-fold serial dilutions ranging from 4 × 10^5 to 2.5 × 10^4 cells on a 24-well plate. Moving from high to low cell density, RECK expression decreased, whereas
miR-7 levels increased. Again, the expression of miR-21 was unaffected by changes in cell density.

During routine cell passage, it was noted that CAL 27 cells generally had stronger adherence to the culture plate and that normal trypsin treatment conditions used for other cancer cell types (e.g., HeLa) would detach only a fraction of CAL 27 cells. Because RECK is required at the cellular level for stable cell substrate adhesion, an experiment was therefore designed to determine whether the collection of detached cells at different time points, (5, 10, and 15 min, during the trypsinization process) would inversely affect the levels of RECK and its regulatory miRNAs. Interestingly, cells that were easily detached at 5 min had significantly lower expression levels of RECK compared with cells that were more adherent and required longer trypsin treatment times (10 and 15 min) to detach. In contrast, the less adherent cells had significantly higher levels of both miR-7 and miR-21 compared with the more adherent cells (Fig. 7C). The expression levels of miR-424, miR-15b, and RNU44, normalized to U6 snRNA, remained unchanged during the differential trypsinization procedure, indicating that the expression changes observed for RECK and the keratinization-associated miRNAs during this process were specific to miR-7 and miR-21. Therefore, the treatment of CAL 27 cells to different physiological conditions further supported an inverse correlation between RECK and keratinization-associated miRNAs.

**DISCUSSION**

In this study, we have identified 69 aberrantly expressed miRNAs in OSCCs in comparison to normal tongue tissues. Because miRNA expressions are known to be tissue- and tumor-specific (35), using the appropriate subset of tumors with the corresponding normal controls is therefore important to reduce the potential complexities associated with analyzing heterogeneous tumor tissues. Thus, our miRNA profiling mainly focused on tongue cancer which is the most prevalent type of oral cancer (36, 37). To date, this is the largest miRNA expression profiling study on tongue cancers that takes into consideration multiple clinicopathological parameters that enable the interpretation of aberrant miRNA expression levels in subtypes of this disease.

Understanding the clinical relevance of miRNA expression patterns in OSCCs is a necessary requirement to better classify these heterogeneous tumors and circumvent the therapeutic challenges faced upon their clinical management. Our data
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have not only shown that the TNM system is a good prognostic parameter, as proposed by Hiratsuka et al. (38), but also illustrated that the keratinization status of OSCCs was closely associated with poor prognosis of patients. Consistent with our findings, several studies have also associated keratinization with carcinogenesis and survival of cancer patients. Keratinization has been associated with the increased incidence of neck metastases and the decreased survival rate of patients with tongue cancer (39). Another study also has demonstrated a significantly poorer survival rate of patients with keratinizing tumors than those with non-keratinizing squamous cell carcinomas, indicating the prognostic importance of keratinization status in tumors of the nasopharynx (40). Altered keratin expression has been observed in the carcinogen-induced hamster cheek pouch carcinogenesis model, which results in the keratinization of squamous cell carcinomas of the oral epithelium, including tongue (39, 41). In agreement with these studies, our findings also indicated that keratinization of tumors was highly associated with poorer survival of patients and that miR-21 could potentially be responsible for this keratinization process.

Our study mainly focused on two miRNAs, miR-7 and miR-21, which were up-regulated in keratinized tumors compared with normal or non-keratinized tumors. Although aberrant expressions of these miRNAs were reported in different types of cancer, this is the first report to identify the link between these miRNAs and keratinization. Overexpression of miR-21 has been associated with poorer survival of patients with tongue squamous cell carcinomas (42). The importance of miR-21 as a poor prognosis indicator, however, is not limited to oral cancer, as similar results have been observed for other types of cancers (30, 32). Recent mechanistic and functional studies focus on the tumor suppressive aspects of miR-7 in cancers (43–46). Although many aberrantly expressed miRNAs have been reported in diverse human cancers, it does not necessarily mean that all of them play a causative role for tumorigenesis. Although the oncogenic miRNAs may induce oncogenesis, some deregulated miRNAs may be the secondary consequence from the loss of normal cellular identity and further contribute to the phenotypic variability of tumors (47). The expression of miR-7 in cancers seems to vary depending on cell or tissue type. For example, miR-7 has been reported to be down-regulated in schwannoma tumors (43) and glioblastoma (48) but up-regulated in breast cancer (49) and lung cancer (50). It is possible that the increase of miR-7 level in tongue tumors examined was due to the consequence or a responsive event during oral carcinogenesis instead of being the cause of cancer development. These observations suggest that although miR-7 act as a tumor suppressive miRNA in normal physiological conditions, the elevation of miR-7 in such environment may contribute to the regulation of a tumor suppressor RECK.

By using in silico analyses, RECK was determined as the best candidate for further investigation among the keratinization-associated miRNA targeted genes because RECK is a key modulator for regulating the extracellular matrix integrity in physiological and pathological states by negatively regulating MMPs (13). In addition to miR-21-mediated regulation of RECK proposed in other studies (51–54), we identified miR-7 as another regulator for RECK and further confirmed that both miRNAs regulate RECK in oral cancer. Although these miRNAs can simultaneously target RECK, our data suggested that different amounts of these miRNAs were required to achieve similar efficacies in target regulation. It is intriguing that RECK has been reported to be down-regulated in tumors of the carcinogen-induced hamster cheek pouch carcinogenesis model (55), which has been known to form keratinized tumors (39, 41). Therefore, the increase in keratinization-associated miR-7 and miR-21 during carcinogenesis could lead to down-regulation of RECK. Together with the deregulated activation of MMPs during keratinization as discussed under “Introduction,” the keratinization-associated miR-7 and miR-21 can help facilitate the aggressiveness of tumors, leading to poor survival.

Our findings show additional evidence that the keratinization-associated miRNAs, miR-7 and miR-21, are inversely correlated with the expression of RECK. CAL 27 orthotopic xenograft tumors are keratinized tumors (26) and so it is a good model to compare the inverse expression patterns between RECK and the keratinization-associated miRNAs. Also, varying the cell density or nutrient supply was a good in vitro system to monitor the inverse correlation between RECK and miRNAs because RECK expression alters upon these changes (33, 34). In both altered conditions, miR-7 appears to act more dynamically to modulate RECK expression in response to environmental changes, and miR-21 is relatively stable in regulating RECK, regardless of the external stimuli. Similar observations were noted on the effects of miR-7 and miR-21 on RECK in CAL 27 or HEK293 cells. Although the regulation of RECK by miR-21 is more likely to be consistent throughout the experiments, miR-7-mediated regulation varies in different assays. For example, miR-7 overexpression mildly changed the endogenous RECK levels, but the repression effects in luciferase assays was stronger than that of miR-21-mediated repression. Western blot analysis and luciferase assays were performed in HEK293 cells to achieve a stronger sensitivity and better transfection efficiency. Undetectable RECK in CAL 27 may, in part, be due to the high expression of these keratinization-associated miRNAs. Together, these data suggest that the increase of keratinization-associated miRNAs posttranscriptionally repress RECK in cancers and that environmental changes may further modulate RECK mRNA levels via changes to specific miRNA levels. In particular, miR-21 may play a more central role in regulating RECK, whereas miR-7 may be more involved in regulating RECK levels on the basis of dynamic changes in tumor microenvironment.

In conclusion, our data imply that the interpretation of miRNA expression patterns can be better resolved when one takes into consideration clinicopathological data of OSCC subtypes. Moreover, our innovative approach of using radar chart analyses provides clearer visualizations of miRNA expression patterns in subtypes of OSCCs. Of significance, our patient survival analyses demonstrated that keratinization and high miR-21 levels were important indicators of oral cancer patient prognosis and that miR-7 and miR-21, two keratinization-associated miRNAs, could contribute to the regulation of the tumor suppressor gene RECK in oral cancers. By understanding the modulation kinetics between keratinization-associated
miRNAs and RECK (which is also involved in the keratinization process), the stimuli that affect their expression levels and the mechanisms of how those molecular events are associated with poor prognosis could ultimately lead to improved therapeutics for oral cancer.

Acknowledgments—We would like to thank members of the Chan laboratory for their technical assistance, discussion, and encouragement. We are grateful to Tissue Procurement at the H. Lee Moffitt Cancer Center for providing the specimens.

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