Downregulated miR329 and miR410 Promote the Proliferation and Invasion of Oral Squamous Cell Carcinoma by Targeting Wnt-7b

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Abstract
microRNA (miRNA) dysregulation contributes widely to human cancer but has not been fully assessed in oral cancers. In this study, we conducted a global microarray analysis of miRNA expression in 40 pairs of betel quid–associated oral squamous cell carcinoma (OSCC) specimens and their matched nontumorous epithelial counterparts. Eighty-four miRNAs were differentially expressed in the OSCC specimens compared with the matched tissue. Among these downregulated miRNAs, 19 miRNAs were found and mapped to the chromosome 14q32.2 miRNA cluster region, which resides within a parentally imprinted region designated as Dlk-Dio3 and known to be important in development and growth. Bioinformatic analysis predicted two miRNAs from the cluster region, miR329 and miR410, which could potentially target Wnt-7b, an activator of the Wnt–β-catenin pathway, thereby attenuating the Wnt–β-catenin signaling pathway in OSCC. Stable ectopic expression of Wnt-7b in OSCC cells overexpressing miR329 or miR410 restored proliferation and invasion capabilities abolished by these miRNA. Combining a demethylation agent and a histone deacetylase inhibitor was sufficient to reexpress miR329, miR410, and Meg3, consistent with epigenetic regulation of these miRNA in human OSCC. Specifically, arecoline, a major betel nut alkaloid, reduced miR329, miR410, and Meg3 gene expression. Overall, our results provide novel molecular insights into how betel quid contributes to oral carcinogenesis through epigenetic silencing of tumor-suppressor miRNA that targets Wnt–β-catenin signaling. Cancer Res; 74(24); 7560–72. ©2014 AACR.

Introduction
Oral cancer, predominantly oral squamous cell carcinoma (OSCC), is the most common head and neck cancer worldwide, with more than 300,000 new cases being diagnosed annually (1, 2). Despite advances in therapy, the 5-year survival rate for OSCC has not improved markedly over the past years, due to late diagnosis and frequent loco-regional recurrences at the primary site and the metastatic neck lymph node after treatment (3, 4). Therefore, molecular signatures that predict disease prognosis and provide a deeper understanding of the molecular mechanisms of OSCC tumorigenesis are urgently needed to improve therapeutic efficacy, as well as to design more effective treatment strategies for OSCC. Epidemiologic studies have shown that tobacco usage, alcohol consumption, betel quid chewing, and human papillomavirus infection are the most common environmental risk factors for the development of oral cancer (5, 6). Among these factors, betel quid has been recognized as the major contributing factor for OSCC in Southeast Asia, including Taiwan (7). However, the molecular mechanisms involved in the pathogenesis of betel quid chewing–associated oral cancer remain largely unknown.

microRNAs (miRNA or miR) are endogenous small noncoding RNAs (18–25 nucleotides) that negatively regulate gene expression at the posttranscriptional level by binding target miRNAs through base pairing to the 3′-untranslated region (3′-UTR) and causing either translational repression or degradation of the miRNA (8, 9). Several mechanisms leading to abnormal expression of miRNAs in cancer have been reported,
such as chromosome rearrangements, genomic copy number change, as well as epigenetic modifications (10–12). More recently, studies have revealed that miRNAs were transcriptionally inactivated by CpG island hypermethylation in several types of cancers (13, 14), suggesting epigenetic modification to be a crucial factor for determining the expression of miRNAs in human cancers.

Here, we present the results of genome-wide miRNA expression profiling in 40 OSCC specimens and their matched non-tumorous epithelial counterparts. Our study identified that a total of 84 miRNAs are differentially expressed in OSCC specimens. We provide data showing that epigenetic silencing of miR329 and miR410, two miRNAs from the 14q32.2 cluster, may contribute to Wnt-7b overexpression and activate the Wnt-β-catenin signaling pathway, thus promoting proliferation and invasion in OSCC tumorigenesis. Finally, the dysregulation of the maternally expressed gene-3 (Meg-3)–miR329 and -410–Wnt-7b–β-catenin signaling axis may result from betel quid chewing.

Materials and Methods

Cell culture, antibodies, vectors, and reagents

Human oral keratinocytes (HOK) were purchased from ScienCell and cultured in oral keratinocyte medium according to the manufacturer’s instructions. OSCC cells, including DOK, FaDu, OC-3, OEC-M1, SCC-4, SCC-9, SCC-15, Tw2.6, and YD-15, were routinely cultured as previously described (15). All cells were authenticated by morphology and growth characteristics, tested for Mycoplasma, and frozen, and cultured at 37°C in a 5% CO2 atmosphere and maintained in 10% FBS (Kibbutz) within 3 months of resuscitation from the frozen stock, with fewer than 20 passages. Wnt-7b antibody (GTX114881) was purchased from Genetex, CCND-1 antibody (2261-S) was purchased from Epitomics, and anti-β-tubulin antibody (MS-581) was purchased from NeoMaker. Antibodies specific for phospho-β-catenin (8814), anti-β-catenin (9582), phospho-serine-9-GSK-3β (9336), GSK-3β (9832), and myc (9420) were purchased from Cell Signaling Technology. The miRNA inhibitors (AM) and miRNA mimics (PM) were chemically modified RNA molecules and obtained from Ambion. Wnt-7b siRNA were purchased from Dharmacon. Human Wnt-7b expression vector was purchased from OriGene. The miRNA overexpression vector pLemiR was kindly donated by Dr. C-C. Chang (Graduate Institute of Oral Biology, School of Dentistry, National Taiwan University, Taipei, Taiwan) (16).

Clinical samples and patient characteristics

Paired tumor specimens and their adjacent non-tumorous epithelia were from patients who underwent curative surgery from 1999 to 2010 at the National Cheng Kung University Hospital (Tainan, Taiwan). Fresh frozen tissues were preserved in liquid nitrogen until use. The American Joint Committee on Cancer staging system was used for tumor staging (17). The study protocol was reviewed and approved by the Institutional Human Experiment and Ethic Committee (HR-97-100).

Microarray profiling

Total RNA and genomic DNA for microarray analysis were isolated from OSCC frozen tissues using the miRNeasy Mini Kit (Qiagen) and QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s protocol. Gene expression profiling was performed using the whole-genome DASL HumanRef-8-v3 chip and miRNA expression profiling was performed using the Human-v2 MicroRNA Expression BeadChips (Illumina, Inc.). Microarray data processing and analysis were done using Illumina BeadStudio software. Microarray data are available in Gene Expression Omnibus (GEO) under accession number GSE37991 for gene expression and GSE45238 for miRNA expression.

Western blot and immunofluorescence assays

Nuclear and cytosolic extracts were obtained according to the REAP method (18) and Western blot analyses were performed as previously described (19). The methods of immunofluorescence staining are summarized in Supplementary Materials and Methods.

Plasmid construction, virus production, and infection of target cells

To generate the sponge-miR329 and miR410 plasmids, a synthetic miRNA sponge sequence containing four specific miRNA binding sites was cloned into the pGIPZ lentivirus plasmid (Open Biosystems). The miRNA-sponge sequences were based on Ebert methods (20) and the bulged sites were predicted with mfold (http://mfold.rna.albany.edu/? q=mfold). The pGIPZ-NS plasmids acted as the negative control in all sponge experiments. The construct of the luciferase reporter, virus production, and infection protocol are described in Supplementary Materials and Methods.

RNA extraction and quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and cDNA was synthesized using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen) for PCR or quantitative PCR (qPCR). For the miRNA assay, specific stem-loop RT primers were designed (21) and used to perform reverse transcription. All primers used in this study are summarized in Supplementary Materials and Methods.

Luciferase assay

The luciferase assay was performed 48 hours after transfection with a control vector or a vector containing part of the 3'-UTR of the Wnt-7b using the Dual-Luciferase assay (Promega) as described by the manufacturer’s protocol. Luminometry readings were obtained using an Orion L luminometer (Berthold).

Functional assays

All OSCC cells used in the functional assays were transfected with the indicated plasmids and stable colonies were selected. The functional assays, such as proliferation and invasion assay, were performed as described previously (15).
For the clonogenic assay, 500 cells were seeded into 6-well plates and cultured for 7 days. Colonies were fixed in 1.0% crystal violet (Merck) and visible colonies were counted.

**Bisulfite sequencing PCR**

Genomic DNA, extracted using the DNA Extraction Kit (Qiagen), was bisulfite-modified by the EZ-DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions. The bisulfite-modified DNA was used to amplify a 640-bp product situated within the Meg-3 promoter region and across exon 1 (−444 to +196 sites) using the Meg-3-CpG1-F and Meg-3-CpG1-R primer sets. The sequenced DNA region for the Meg-3 promoter was confirmed using an automated sequencer (ABI automated sequencer).
miR329 and miR410 directly target Wnt-7b. A, comparison of nucleotides in the miR329 and miR410 seed sequence and the 3'-UTR region of Wnt-7b. B, the expression levels of miRNAs in OSCC cell lines compared with HOK by qRT-PCR. *** P < 0.001 versus HOK. C, the expression level of Wnt-7b by RT-PCR and Western blot analysis (W.B.). D, the effect of miR-PM (100 nmol/L) on the luciferase activities of the constructs containing the wild-type (wt) or mutant-type (mt) 3'-UTR fragments in OEC-M1 cells. The relative luciferase activities are the ratios of Renilla luciferase normalized to the control mimics. The data are represented as mean ± SD; *** P < 0.001 versus control. E, Western blot analysis of Wnt-7b expression in the indicated cells transfected with 100 nmol/L of PM (OEC-M1) or AM (OC-3). F, the expression level of miR329 (n = 62) and miR410 (n = 66) in OSCC tumors (T) compared with their own adjacent normal tissues (N). *** P < 0.001. G, the correlation between Wnt-7b and miR329 (left) or miR410 (right) in patients with OSCC (n = 28) by qRT-PCR analysis.
miR329 and miR410 modulate the Wnt-β-catenin signaling pathway. A, dual-luciferase assay showing Top-Flash/Fop-Flash reporter activity following treatment with LiCl for 6 hours (left), miR-PM (middle), or miR-AM (right) for 24 hours in OEC-M1 cells. The values were normalized to a Renilla transfection control. Three independent assays were performed and are represented as mean ± SD. ***, P < 0.01; ****, P < 0.001. B, a Western blot analysis showing active β-catenin translocation by PM treatment in OEC-M1 cells. α-Tubulin and histone H3 were used as loading controls. C and D, Western blot analysis showing the effects of miR-PM (100 nmol/L) in OEC-M1 cells, miR-AM (100 nmol/L) in OC-3 cells, or two target-specific Wnt-7b siRNA (20 nmol/L) in OEC-M1 cells on the Wnt-β-catenin signaling pathway. E, Top-Flash/Fop-Flash assays quantifying relative si-Wnt-7b signaling activity in OEC-M1 cells. F, cellular distribution of β-catenin in OEC-M1 cells transfected with either 100 nmol/L of miR-control (NS) or 100 nmol/L of miR-PM for 48 hours. Scale bar, 50 μm.
Animal studies

OEC-M1 or SCC-15 cells were transfected with the indicated miRNA-expressing plasmids and stable colonies were selected. In vivo studies were performed as described in Supplementary Materials and Methods. All mice were purchased from the National Laboratory Animal Center (Taiwan) and experiments were performed in strict accordance with the recommendations in the guidelines for the Care and Use of Laboratory Animals of the National Health Research Institutes (Miaoli, Taiwan). The protocol was approved by the Institutional Animal Care and Use Committee of the National Health Research Institutes.

Immunohistochemistry

For immunohistochemical study of tissue Wnt-7b, 83 oral cancer specimens from previously untreated patients with OSCC, who received curative surgery as their main treatment modality at the National Cheng Kung University Hospital between 1999 and 2010, were included. Immunodetection was performed with a standard LSAB 

++ detection kit (DakoCytomation) and incubated with anti-Wnt-7b antibody (R&D Systems; AF3460). Tumor Wnt-7b levels were scored according to the Wnt-7b staining intensity as follows: 0, negative; 1, weak; 2, intermediate; and 3, strong.

Figure 4. The effects of miR329 and -410 or Wnt-7b on tumor cell proliferation. A and D, qRT–PCR analysis showing the expression level of miR329 and miR410 in OEC-M1 (A) and OC-3 (D) stable cell lines. B and E, the MTT assay showing the relative proliferation in OEC-M1 (B) and OC-3 (E) stable cell lines. C, proliferation assay for restored Wnt-7b in miR329- and miR410-overexpressing OEC-M1 stable cell lines. F, proliferation assay for siWnt-7b in miR329- and miR410-knockdown OC-3 stable cell lines. G and H, monolayer colony formation assays showing the effect of miR329 and miR410 overexpression (G) or restoration of Wnt-7b in the OEC-M1- and SCC-15-overexpressing stable cell lines (H). I, Transwell invasion assays showing the effect of miR329, miR410, or restored Wnt-7b in the OEC-M1- and SCC-15-overexpressing stable cell lines. All data are represented as mean ± SD; ++, \( P < 0.01 \) and +++, \( P < 0.001 \).

miR329 and miR410 Regulate Wnt–β-Catenin Signaling Pathway
Statistical analysis
Between-group differences were analyzed by the two-tailed Student t test. All statistical analyses were performed using GraphPad Prism Ver. 4.01; P < 0.05 denoted statistical significance.

Results
Global miRNA profiling of OSCC patients
To identify deregulated miRNAs in oral cancer, miRNA expression profiles were generated from 40 pairs of OSCC specimens (Supplementary Table S1) and their corresponding nontumorous epithelia by using a custom microarray platform. We further identified 84 miRNAs that were differentially expressed with >2-fold changes (P < 0.05) between the 40 pairs of OSCC specimens (Supplementary Fig. S1A and Supplementary Table S2), wherein there were 32 miRNAs with increased expression and 52 miRNAs with decreased expression. Interestingly, a large number of 19 downregulated miRNAs were mapped on the chromosome 14q32.2 region and resided within a parentally imprinted Dlk1–Dio3 region on chromosome 14q32.2 (Supplementary Fig. S1B).
The expression intensity of 66 mature miRNAs, which was encoded from the chromosome-14q32.2, was arranged according to fold change by microarray analysis (Supplementary Fig. S1C).

Coordinate downregulation of cluster miRNAs and noncoding transcripts in the Dlk1–Dio3 region at 14q32.2

Meg-3 represents a large noncoding RNA as the initial transcript of the Dlk1–Dio3 locus (22). The loss of Meg-3 and other neighboring genes have altered expression levels, we performed a genome-wide expression array using the same set of 40 pairs from the OSCC specimens. Meg-3 expression was significantly downexpressed as were the other neighboring protein-coding genes, Dlk1 and Dio3 (Fig. 1A). Using qRT-PCR, we verified that the expression level of Meg-3 transcript was markedly reduced in patients with OSCC (Fig. 1B) and in OSCC cell lines compared with the normal keratinocyte HOK (Fig. 1C and D). Furthermore, we also observed significant downexpression of the 14q32.2 miRNAs in patients with OSCC and cell lines (Fig. 1E and F). These results indicate that the expression of the Meg-3 and cluster miRNAs, which locate on the Dlk1–Dio3 imprinted region, is coordinately regulated in OSCC.

miRNAs target the activator of the Wnt signaling pathway

The Wnt signaling pathway has been reported to be significantly enriched in the Meg3-null mice by microarray analysis (25). We also found that many molecules differentially expressed in our expression array are enriched in the Wnt–β-catenin signaling pathway, including Wnt-7b protein. To test whether Wnt-7b was targeted by some 14q32.2 miRNAs, we used targeting algorithms (TargetScan and microRNA.org) combined with microarray data to search for putative miRNAs that might bind to Wnt-7b mRNA. We identified two miRNAs, miR329 and miR410, which could potentially target Wnt-7b. Figure 2A illustrates the predicted miRNA binding sites in the 3’-UTR of Wnt-7b. A significantly negative correlation between the miRNAs and the Wnt-7b was found in OSCC cells (Fig. 2B and C). To validate miRNA–target protein interactions, the Wnt-7b 3’-UTR fragment, containing the wild-type or mutant miRNA binding sequence, was cloned into the Renilla luciferase reporter and then cotransfected with miR329 or miR410 mimics (PM) into OEC-M1 cells. Both miRNAs significantly reduced the luciferase activity with respect to the scrambled sequence; however, mutations in the miR329 or miR410 binding sites resulted in a complete reversal of the luciferase activity (Fig. 2D). Subsequently, the overexpression of individual miR-PM induced a marked reduction of Wnt-7b protein levels (Fig. 2E, left). Otherwise, the depletion of miR329 or miR410 with miRNA inhibitor (AM) caused the upregulation of Wnt-7b (Fig. 2E, right), establishing Wnt-7b as a target of both miR329 and miR410. However, there is no toxic effect in our miRNA overexpression system (Supplementary Fig. S2). To consolidate our findings, we correlated the expression level of miRNAs (Fig. 2F) to Wnt-7b mRNA using clinical OSCC specimens and found a strong inverse correlation between the expression levels of miR329 or miR410 and Wnt-7b (Fig. 2G).
Figure 7. The effects of epigenetic modifiers on the reexpression of Meg-3 and miRNAs. A, methylation pattern of the CpG island (blue box) in the meg-3 promoter. The depicted region corresponds to a 680-bp section across the transcriptional start site (indicated by arrow). Vertical bars represent CpG-dinucleotides. The methylation status in OSCC cells is shown by a BSP assay. (Continued on the following page.)
miR329 and miR410 modulate the Wnt–β-catenin signaling pathway

Because miR329 and miR410 directly target Wnt-7b, it suggested the possibility that these two miRNAs might regulate Wnt–β-catenin signaling. To test this hypothesis, the Wnt signaling reporter was validated by Top-Flash/Fop-Flash assay (16). LiCl was used to stimulate Wnt–β-catenin signaling (26) and acts as a positive control (Fig. 3A, left). Overexpression of miR-PMs suppressed TCF/LEF1 transcriptional activity (Fig. 3A, middle); in contrast, the overexpression of miR-AM increased TCF/LEF1 transcriptional activity (Fig. 3A, right). Moreover, the overexpression of miR-PMs resulted in a decreased amount of active nuclear β-catenin in nucleus (Fig. 3B).

Next, we introduced cells with miR329 or miR410 and examined the phosphorylation status of β-catenin (active) and GSK-3β (inactive). As expected, miR-PMs markedly suppressed the phosphorylation of GSK-3β and β-catenin in OEC-M1 cells, consequently, causing a downregulation of cyclin D1 and c-Myc (Fig. 3C) and increase in β-catenin translocation to the cell membrane (Fig. 3F). Similar results were observed for another SCC-15 cell line (Supplementary Fig. S3A). Conversely, the silencing of endogenous miRNAs with miRNA-AMs caused an opposite effect in OC-3 and SCC-4 cells (Fig. 3C and Supplementary Fig. S3A). To further investigate the impact of Wnt-7b on the Wnt–β-catenin signaling pathway, we used two target-specific siRNAs specifically targeting Wnt-7b. A marked decrease in inactive GSK-3β, active β-catenin, c-Myc, and cyclin D1 was observed after Wnt-7b knockdown (Fig. 3D). Most notably, the si-Wnt-7b overexpression suppressed TCF/LEF1 transcriptional activity (Fig. 3E) and increased β-catenin translocation to the cell membrane (Supplementary Fig. S3B).

miR329 and miR410 reduce the proliferation and invasiveness of OSCC cells

To understand the biologic functions of miR329 and miR410 in OSCC cells, we then transfected OSCC cell lines with miRNA expression vector (pLemiR) or miRNA-sponge vector. We found that miR329 or miR410 in OEC-M1 stable cells was significantly increased when compared with the corresponding pLemiR-control cells (Fig. 4A). The tumor cell proliferation, monolayer colony formation, and invasive ability were notably decreased in OEC-M1 stable cells that were overexpressing miR329 or miR410 (Fig. 4B and G–I). To determine whether Wnt-7b acts as a functional target of miR329 or miR410 in OSCC cells, we transferred a vector-based Wnt-7b without 3'-UTR into miR329- or miR410-overexpressing stable cells (Supplementary Fig. S4A). Proliferation, colony formation, and invasion abilities were recovered after exogenous expression of Wnt-7b in miRNA overexpression in stable cells (Fig. 4C, H, and I). As expected, tumor cell proliferation and monolayer colony formation were significantly increased in OC-3 cells that were stably expressing the miR329 and miR410 sponge vector (Fig. 4D–F). Importantly, the knockdown of Wnt-7b in the miRNA-sponge stable OC-3 cells could totally block miR329- and miR-410-induced proliferation and monolayer colony formation (Fig. 4F and Supplementary Fig. S4B–S4D). Furthermore, overexpression of miR-PM in OSCC cell lines has a dose-dependent pattern on secreted Wnt-7b protein level in culture medium (Supplementary Fig. S4E).

Effects of miR329 and miR410 suppress tumorigenicity in a xenograft model

To further confirm the above findings, an in vivo xenograft mouse model was carried out by subcutaneous injection of miRNA-overexpressing stable OEC-M1 cells (Fig. 5A and D). As compared with the vector control (NS) group, the average tumor weight (Fig. 5B and E) and the average tumor volume (Fig. 5C and F) were significantly reduced in the miRNA overexpression group. Moreover, we detected the expression of miRNA, Wnt-7b, and active-β-catenin in the harvested tumor tissues. Similar to the in vitro results, the levels of miRNAs were significantly increased in the tumors derived from miRNA-overexpressing cells (Supplementary Fig. S5). All the expression levels of the Wnt-7b and active-β-catenin proteins were decreased in the miRNA overexpression groups (Fig. 5G and H). Similar results are represented in the SCC-15 xenograft experiment (Supplementary Fig. S6).

Wnt-7b correlates with lymphovascular invasion in OSCC

To explore the potential role of Wnt-7b, we first investigated the expression patterns of the Wnt-7b in 83 OSCC specimens, and then correlated these with the clinical parameters of these patients. Figure 6A shows that Wnt-7b was mainly expressed by the less-differentiated basal cells (top right, arrowheads) in the adjacent nontumorous epithelium, but was diffusely expressed in the underlying infiltrating tumor cells (top right, "Tumor"). Moreover, for each individual tumor nest, Wnt-7b was mainly expressed by the less differentiated tumor cells (bottom left, arrowheads). The keratinized, more differentiated tumor cells in the center of the tumor nests showed less Wnt-7b expression (** in the bottom left), suggesting that Wnt-7b may correlate with cellular differentiation (Supplementary Table S3). High tumor Wnt-7b also significantly correlated with pathologic lymphovascular invasion (Supplementary Table S3).
Table S3). We further subdivided the 83 patients with OSCC into the low tumor Wnt-7b group (n = 25; score 0 and 1) and the high tumor Wnt-7b group (n = 58; score 2 and 3) according to their tumor Wnt-7b staining intensity scores (Fig. 6A, bottom right). When survival was compared between these two groups (Fig. 6B), patients with high Wnt-7b expression tended to have a less favorable relapse-free survival and had a significantly poorer disease-specific survival rate compared with patients with low tumor Wnt-7b expression.

**Loss of MEG-3 and its encoded miRNAs in OSCC cells with epigenetic regulation**

The results described above indicate that the dysregulation of miR329 and miR410 has a profound impact on multiple functions in OSCC tumorigenesis; however, little is known about the regulatory mechanisms. Recent reports showed that epigenetic modification of the MEG-3 differentially methylated region (Meg-3-DMR) in inactivation of the MEG-3 and 14q32.2 miRNAs may play a significant role in human tumors (24, 27). Computational analysis identified a putative CpG island across the transcriptional start site of the MEG-3 transcript (Fig. 7A) and overlaps with the MEG-3-DMR (28). To establish the precise epigenetic status of individual CpGs within the MEG-3-DMR region, bisulfite sequencing was performed. All three OSCC cell lines were found to have hypermethylation of the MEG-3-DMR; the methylation status could be reversed by treatment with 5-Aza (DNA methyltransferase inhibitor), which resulted in the reexpression of the MEG-3 transcript (Fig. 7B), miR329 and miR410 (Fig. 7C). We further analyzed the degree of methylation of the MEG-3-DMR in 11 patients with OSCC; the average methylation percentage of the MEG-3-DMR in tumors (86.1%) was higher than that (68%) in their adjacent normal tissue (Fig. 7D). Next, we determined the effect of arecoline, a major component of betel nut alkaloids, on the expression of MEG-3 and the 14q32.2 miRNAs. We found that arecoline treatment could reduce the expression level of MEG-3 and the 14q32.2 miRNAs in a time-dependent pattern in HOK cells (Fig. 7E and F). Similar to the effect of miR-AM, arecoline treatment triggered the upregulation of Wnt-7b and markedly enhanced the phosphorylation of GSK-3β and active-β-catenin in DOK cells, consequently causing an upregulation of cyclin D and c-Myc (Supplementary Fig. S7A). Furthermore, miR-PM could decrease the arecoline-induced proliferation rate in DOK cells (Supplementary Fig. S7B). Using gene set enrichment analysis to predict transcription factor in miRNA overexpression OEC-M1 cells, we significantly enriched the CTNNB1 (β-catenin) in our transcription factor category (Supplementary Table S5). Taken together, these results emphasize that betel quid may contribute to oral carcinogenesis through the silencing of tumor-suppressor miRNAs, consequently activating the Wnt–β-catenin signaling pathway.

**Discussion**

Aberrant miRNA expression patterns have been widely reported in various cancer types (29–32). In this study, we identified a large group of OSCC-associated miRNA clusters located on the chromosome 14q32.2 region and within a parentally imprinted domain designated Dlk1-Dio3 (33). Meg-3 represents an IncRNA as its transcript from the Dlk1-Dio3 region that could initiate at the Meg-3 promoter (22). The loss of Meg-3 expression has been found in various types of tumors (23, 24), which is caused by the loss of gene copy number and/or CpG methylation (22). Three pieces of evidence support the epigenetic regulation mechanism of Meg-3 expression in our OSCC model. First, bisulfite sequencing PCR (BSP) analysis confirmed an increased methylation of the CpG islands within the Mrg-3-DMR in OSCC cells (95.5%–97.1%) in comparison with the normal HOK (40.71%; Fig. 7A and B), which is responsible for the expression of Meg-3 (Fig. 2C). Second, the treatment of OSCC cells with a demethylating agent alone enhanced Meg-3 expression more than with HDAC inhibitor alone (Fig. 7B). Third, the regulation of imprinting genes in this region is thought to be tightly mediated by an intergenic differentially methylated region (IG-DMR) and Meg-3-DMR (28). This indicates that Meg-3-DMR and IG-DMR function as imprinting control centers with a hierarchical interaction for a methylation pattern (28). Taken together, our data indicate that Meg-3 can be modulated by a methylation mechanism in OSCC cells, consistent with other reports (34–36). In addition, it is important to emphasize that the expression of the 14q32.2 miRNAs and Meg-3 transcripts is regulated by the DMR methylation status (37, 38), which results in DNA conformation change and long-range epigenetic silencing in this region. However, the regulation of miRNAs from this cluster is complex and needs to be explored further.

Meg-3 knockout mice have been generated to identify targets and potential functions of this noncoding gene in embryonic development and tumorigenesis (25). Several pathways are significantly enriched in the Meg-3-null mice, such as MAPK, Notch, VEGF, and Wnt signaling pathways. Constitutive activation of the Wnt signaling pathway is a common feature of solid tumors and contributes to tumor development and progression in various cancers (39, 40). Loss of Meg-3 also leads to upregulation of Wnt signaling pathway–related genes, such as Wnt-5a, Wnt-5b, and Wnt-7b (25), which implies that the Meg-3 transcript or its encoded miRNA cluster may play some important roles in regulating the Wnt pathway. Here, we identified Wnt-7b as a direct target of miR329 and miR410 in oral cancer cells through translational inhibition. There are few reports discussing the cellular functions of miR329 in mammalian cells. However, miR410 has been reported to function as a tumor suppressor in human gliomas (41). Our rescue experiment showed that the reintroduction of Wnt-7b in miR329- or miR410-overexpressing OSCC cells antagonized the effects of miR329 and miR410 on growth inhibition (Fig. 4), confirming our conclusion that Wnt-7b is a functional mediator for miR329 and miR410 in OSCC cells. However, patients with high-level miR329 or miR410 expression tended to have a less favorable relapse-free survival and disease-specific survival rates compared with patients with low tumor miRNA expression (data not shown). The poor result may be due to the smaller sample size. However, Wnt-7b has a strong inverse correlation between the expression levels of miR329 and...
miR329 and miR410 in OSCC specimens (Fig. 2G). These two miRNAs also regulate Wnt–β-catenin signaling in in vitro and in vitro models (Figs. 3 and 5), suggesting that miR329 and miR410 still play an important role in OSCC carcinogenesis.

As mentioned above, this study showed that the loss of Meg-3 transcript and 14q32.2 miRNA expression was significantly correlated with Meg-3 promoter hypermethylation (Fig. 7A–C). Thus far, the reasons for Meg-3 promoter hypermethylation during oral carcinogenesis are not clear. Betel quid chewing is well recognized as one of the most important environmental risk factors for oral carcinogenesis (42–44). In this study, we identified miR329 and miR410 that were consistently downregulated in accordance with Meg-3 expression following arecoline exposure in HOK cells (Fig. 7E and F), suggesting that betel nut chewing may contribute to the downregulation of Meg-3 and the partial of 14q32.2 miRNAs. Several lines of evidence indicate that hypermethylation may be involved in the pathogenesis of oral cancer associated with betel quid chewing (45, 46). Therefore, it is reasonable to suppose that the silencing of the 14q32.2 miRNAs, which would seem to be correlated with Meg-3 promoter hypermethylation, is related to betel quid chewing and might be a significant event in oral carcinogenesis.

In conclusion, our findings define the relationship between the miR329/miR410 and the Wnt–β-catenin pathway, implying functional mechanisms in oral carcinogenesis. Importantly, the dysregulation of the Meg-3/miR329 and –410–Wnt-7b–β-catenin signaling axis may result from exposure to betel quid chewing (Fig. 7G). To the best of our knowledge, this is the first investigation suggesting that betel quid chewing has a significant impact on Meg-3/miR329 and –410 expression in OSCC. A further study focusing on these alternative mechanisms is now warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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