Downregulated miR-329 and miR-410 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 non-tumorous epithelial counterparts. Eighty-four miRNAs were differentially expressed in the OSCC specimens compared to 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 Dlk-Dio3 and known to be important in development and growth. Bioinformatic analysis predicted two miRNAs from the cluster region, miR-329 and miR-410, 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 miR-329 or miR-410 restored proliferation and invasion capabilities abolished by these miRNA. Combining a demethylation agent and a histone deacetylase inhibitor was sufficient to re-express miR-329, miR-410 and Meg3 consistent with epigenetic regulation of these miRNA in human OSCC. Specifically, arecoline, a major betel nut alkaloid, reduced miR-329, miR-410 and Meg3 gene expression. Overall, our results provide novel molecular insights into how betel quid
contribute to oral carcinogenesis through epigenetic silencing of tumor suppressor miRNA which target Wnt/β-catenin signaling.
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 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 non-coding RNAs (18~25 nucleotides) that negatively regulate gene expression at the post-transcriptional level by binding target mRNAs through base pairing to the 3′-untranslated region (3′-UTR).
and causing either translational repression or degradation of the mRNA (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, few evidences 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 provides data showing that epigenetic silencing of miR-329 and miR-410, two miRNAs from 14q32.2 cluster, may contribute to Wnt-7b overexpression and to activate Wnt/β-catenin signaling pathway, thus promoting proliferation and invasion in OSCC tumorigenesis. Finally, the dysregulation of the maternally expressed gene-3 (Meg-3)/miR-329&410/Wnt-7b/β-catenin signaling axis may result from exposure to betel quid chewing.
Materials and Methods

Cell culture, antibodies, vectors, and reagents

Human oral keratinocytes (HOK) was purchased from ScienCell (Carlsbad, CA, USA) and cultured in oral keratinocyte medium (Carlsbad, CA, USA) according to the manufacturer’s instructions. OSCC cells, including DOK, FaDu, OC-3, OEC-M1, SCC-4, SCC-9, SCC-15, SCC-25, 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% fetal bovine serum (FBS, Kibbutz BeitHaemek, Israel) within 3 months of resuscitation from the frozen stock, with lower than 20 passages. Wnt-7b antibody (GTX114881) was purchased from Genetex, CCND-1 antibody (2261-S) was purchased from Epitomics, and anti-α-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. The miRNA inhibitors (AM) and miRNA mimics (PM) were chemical 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 over-expression vector pLemiR was obtained from Open-Biosystem. TOP/FOP flash TCF reporter vectors were kindly donated by Dr. C-C. Chang (16).
Clinical samples and patient characteristics

Paired tumor specimens and their adjacent non-tumorous epithelia were received from curative surgery from 1999 to 2010 at the National Cheng Kung University Hospital. 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 GEO under accession number GSE37991 for genes 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 blots were performed as previous described (19). The methods of
immunofluorescence staining are summarized in the “Supplementary Materials and Methods”.

**Plasmids construction, virus production, and infection of target cells**

To generate the sponge-miR-329 and -410 plasmids, a synthetic miRNA sponge sequence containing 4 specific miRNA binding sites was cloned into the pGIPZ lentivirus plasmid (Open-Biosystem). The miRNA-sponge sequences were based on Ebert’s 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 the “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 the “Supplementary Materials and Methods”.

**Luciferase assay**


The luciferase assay was performed 48 h post 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 (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 (BSP)**

Genomic DNA, extracted using the DNA extraction kit (Qiagen), was bisulfite-modified by 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 cross 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).

**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 National Health Research Institutes, 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 OSCC patients, who received curative surgery as their main treatment modality at the National Cheng Kung University Hospital from 1999 to 2010, were included. Immunodetection was performed with a standard LSAB⁺ detection kit (DakoCytomation) and incubated with anti-Wnt-7b antibody (R&D 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.

**Statistical analysis**

Between-group differences were analyzed by 2-tailed Student’s t test. All statistical analyses were performed using GraphPad Prism Ver. 4.01 (San Diego, CA); p<0.05
was denoted a statistical significance.

**Results**

**Global miRNA profiling of OSCC patients.**

In order to identify deregulated miRNAs in oral cancer, miRNA expression profiles were generated from 40 pairs of OSCC specimens (Supplementary Table S1) and their corresponding non-tumorous 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; Supplementary Table S2), wherein 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 miRNAs cluster and non-coding transcripts in the Dlk1-Dio3 region at 14q32.2**
Meg-3 represents a large noncoding RNA as the initial transcript of Dlk1–Dio3 locus (22). The loss of Meg-3 expression and chromosome-14q32.2 miRNAs were consistently found in various types of tumors (23, 24). In order to determine whether 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 well as 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 OSCC patients (Fig.1B) and in OSCC cell lines compared to the normal keratinocyte HOK (Fig.1C; Fig.1D). Furthermore, we also observed significant downexpression of the 14q32.2 miRNAs in OSCC patients and cell lines (Fig.1E; Fig.1F). These results indicate that the expression of the Meg-3 and cluster miRNAs which locate on the Dlk1-Dio3 imprinted region are coordinately regulated in OSCC.

miRNAs target the activator of the Wnt signaling pathway.

Wnt signaling pathway has been reported significantly enriched in the Meg3-null mice by microarray analysis (25). We also found that much of molecules with differential expressed in our expression array are enriched in Wnt/β-catenin signaling pathway, including Wnt-7b protein. To test whether Wnt-7b was targeted by some of
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, miR-329 and miR-410, could potentially target Wnt-7b. Fig.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; Fig.2C). 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 miR-329 or miR-410 mimics (PM) into OEC-M1 cells. Both miRNA significantly reduced the luciferase activity with respect to the scrambled sequence; however, mutations in the miR-329 or miR-410 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 panels). Otherwise, the depletion of miR-329 or miR-410 with miRNA inhibitor (AM) caused the upregulation of Wnt-7b (Fig.2E, right panels), establishing Wnt-7b as a target of both miR-329 and miR-410. 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 specimen and found a strong inverse correlation
between the expression levels of miR-329/or miR-410 and Wnt-7b (Fig.2G).

**miR-329 and -410 modulate the Wnt/β-catenin signaling pathway.**

Because miR-329 and miR-410 directly target Wnt-7b, 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 miR-329 or miR-410 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, caused 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 miR-AMs caused an opposite
effects in OC-3 and SCC-4 cells (Fig.3C; 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 were observed after Wnt-7b knockdown (Fig.3D). Most notably, the si-Wnt-7b overexpression suppressed TCF/LEF1 transcriptional activity (Fig.3E) and increase in β-catenin translocation to the cell membrane (Supplementary Fig.S3B).

**miR-329 and miR-410 reduce the proliferation and invasiveness of OSCC cells.**

To understand the biological functions of miR-329 and miR-410 in OSCC cells, we then transfected OSCC cell lines with miRNA expression vector (pLemiR) or miRNA-sponge vector. We found that miR-329 or miR-410 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 miR-329 or miR-410 (Fig.4B; Fig.4G; Fig.4H; Fig.4I). To determine if Wnt-7b acts as a functional target of miR-329 or miR-410 in OSCC cells, we transferred a vector-based Wnt-7b without 3’-UTR into miR-329- or miR-410-overexpressing stable cells (Supplementary Fig.S4A). The ability of
proliferation, colony formation, and invasion were recovered after exogenous expression of Wnt-7b in miRNA-overexpression stable cells (Fig.4C; Fig.4H; Fig.4I).

As expected, tumor cell proliferation and monolayer colony formation were significantly increased in OC-3 cells that were stably expressing the miR-329 and miR-410 sponge vector (Fig.4D; Fig.4E; Fig.4F). Importantly, the knockdown of Wnt-7b in the miRNA-sponge stable OC-3 cells could totally block miR-329- and miR-410-induced proliferation and monolayer colony formation (Fig.4F; Supplementary Fig.S4B-S4D). Furthermore, overexpression of miR-PM in OSCC cell lines have a dose-dependent pattern on secreted Wnt-7b protein level in culture medium (Supplementary Fig.S4E).

**Effects of miR-329 and miR-410 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; Fig.5D). As compared with the vector control (NS) group, the average tumor weight (Fig.5B; Fig.5E) and the average tumor volume (Fig.5C; Fig.5F) 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). The expression levels of the Wnt-7b and active-β-catenin proteins were all decreased in the miRNA overexpression groups (Fig. 5G; Fig. 5H). Similar results are represented in 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 with the clinical parameters of these patients. Fig. 6A shows that Wnt-7b was mainly expressed by the less-differentiated basal cells (right upper panel, arrow heads) in the adjacent non-tumorous epithelium, but was diffusely expressed in the underlying infiltrating tumor cells (right upper panel, “Tumor”). Moreover, for each individual tumor nest, Wnt-7b was mainly expressed by the less differentiated tumor cells (left lower panel, arrow heads). The keratinized, more differentiated tumor cells in the center of the tumor nests showed less Wnt-7b expression (left lower panel, asterisks**), suggesting that Wnt-7b may correlate with cellular differentiation (Supplementary Table S3). High tumor Wnt-7b also significantly correlated with pathological lymphovascular invasion (Supplementary Table S3). We further subdivided the 83 OSCC patients 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, right lower panel). When survival was compared between these 2 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 to 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 miR-329 and miR-410 has profound impacts 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 (Meg3-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 3 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 re-expression of the Meg-3 transcript (Fig. 7B), miR-329 and miR-410 (Fig. 7C). We further analyzed the degree of methylation of the Meg-3-DMR in 11 OSCC patients; 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; Fig. 7F). 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, caused 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 a lncRNA 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, 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; Fig.7B), 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 and consistent with other reports (34-36). Additionally, it is important to emphasize that the expression of the 14q32.2 miRNAs and Meg-3 transcripts are regulated by the DMR methylation status (37, 38), which result 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.

Meg3 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 Meg3-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 Wnt pathway. Here, we identified Wnt-7b as a direct target of miR-329 and miR-410 in oral cancer cells through translational inhibition. There are not many literatures to discuss the cellular functions of miR-329 in mammalian cells. However, miR-410 has been reported to function as a tumor suppressor in human gliomas (41). Our rescue experiment showed that the
reintroduction of Wnt-7b in miR-329- or miR-410-overexpressing OSCC cells antagonized the effects of miR-329 and miR-410 on growth inhibition (Fig.4), confirming our conclusion that Wnt-7b is a functional mediator for miR-329 and miR-410 in OSCC cells. Although patients with high level miR-329 or miR-410 expression tended to have a less favorable relapse-free survival and disease-specific survival rate compared to 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 miR-329 and miR-410 in OSCC specimen (Fig.2G). These two miRNA also regulate Wnt/β-catenin signaling in vitro and in vitro model (Fig.3 and Fig.5), suggesting that miR-329 and miR-410 still playing 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-7C). Thus far, it is not clear what reasons are for Meg-3 promoter hypermethylation during oral carcinogenesis. Betel quid chewing is well recognized as one of the most important environmental risk factors for oral carcinogenesis (42-44). In the present study, we identified miR-329 and miR-410 that were consistently downregulated in accordance with Meg-3 expression following
arecoline exposure in HOK cells (Fig. 7E; Fig. 7F), 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 miR-329/-410 and the Wnt/β-catenin pathway, which implies functional mechanisms in oral carcinogenesis. Importantly, the dysregulation of the Meg-3/miR-329&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/miR-329&410 expressions in OSCC. A further study focusing on these alternative mechanisms is now warranted.

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References:


FIGURE LEGENDS

Figure 1. The expression pattern of Meg-3 and miRNAs in OSCC patients and cell lines.

A. Microarray analysis of Meg-3 expression and the other neighboring protein-coding genes, Dlk-1 and Dio-3, in 40 OSCC tissue pairs. Red indicates overexpression; green represents downexpression.

B. The expression levels of Meg-3 in OSCC patients (n=15).

C. and D. Meg-3 expression level in 8 cell lines using qRT-PCR and RT-PCR.

E. and F. The expression levels of 6 miRNAs from the chromosome 14q32.2 cluster in 10 OSCC patients and 8 cell lines using qRT-PCR. All data are represented as mean±SD, **p<0.01 and ***p<0.001.

Figure 2. miR-329 and miR-410 directly target Wnt-7b.

A. Comparison of nucleotides in the miR-329 and miR-410 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 (W.B.).

D. The effect of miR-PM (100 nM) 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 nM of PM (OEC-M1) or AM (OC-3).

F. The expression level of miR-329 (n=62) and miR-410 (n=66) in OSCC tumors (T) compared with their own adjacent normal tissues (N). ***p<0.001.

G. The correlation between Wnt-7b and miR-329 (left) or miR-410 (right) in OSCC patients (n=28) by qRT-PCR analysis.

Figure 3. miR-329 and miR-410 modulate the Wnt/β-catenin signaling pathway.

A. Dual luciferase assay showing Top-Flash/Fop-Flash reporter activity following treatment with LiCl for 6 h (left), miR-PM (middle) or miR-AM (right) for 24h in OEC-M1 cells. The values were normalized to a Renilla transfection control. Three independent assays were performed and represented as mean ± SD. ***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 showing the effects of miR-PM (100 nM) in OEC-M1 cells, miR-AM (100 nM) in OC-3 cells or two target-specific Wnt-7b siRNA (20 nM) 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 nM of miR-control (NS) or 100 nM of miR-PM for 48 hours. Scale bar, 50 μm.

Figure 4. The effects of miR-329 and -410 or Wnt-7b on tumor cell proliferation.

A. and D. qRT–PCR analysis showing the expression level of miR-329 and -410 in OEC-M1(A) and OC-3 (D) stable cell lines.

B and E. 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 miR-329- and miR-410-overexpressing OEC-M1 stable cell lines.

F. Proliferation assay for siWnt-7b in miR-329- and miR-410-knockdown OC-3 stable cell lines.

G. and H. Monolayer colony formation assays showing the effect of miR-329 and miR-410 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 miR-329, -410 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 ***$p<0.001$.

**Figure 5. Effects of miR-329 and -410 on tumorigenicity.**

**A. and D.** In vivo image detection of the xenograft tumor growth on miR-329 (A) or miR-410 (D) overexpression OEC-M1. The tumor images were also shown in the right panel by photography. NS means mock control. Mouse #6 and #12 were omitted in photography because of instrument capacity.

**B. and E.** Tumor weight was measured by necropsy.

**C. and F.** Tumor burden was measured and calculated every 3 days on miR-329 (C) or miR-410 (F) overexpression tumor.

**G. and H.** Western blot analysis of Wnt-7b (G) or active-β-catenin (H) in miR-329 overexpression (upper) or miR-410 overexpression (lower) xenograft tumor tissue. α-Tubulin was used as a loading control. All data are represented as mean±SD, ***$p<0.001$ versus NS control.

**Figure 6. Wnt-7b correlates with clinical outcome in OSCC tissue.**

**A.** Immunohistochemical study of Wnt-7b expression in 83 human OSCC specimens.

*Left upper panel*, H&E staining of a representative OSCC specimen, showing both
adjacent normal oral epithelium (Epi) and underlying tumor nests (Tumor). **Right upper panel,** Wnt-7b staining on the same specimen, demonstrating strong Wnt-7b expression at the basal cell layer of the adjacent normal oral mucosa (arrowheads) and in the infiltrating tumors cells (Bar, 200 μM). **Left lower panel,** a higher magnification image (Bar, 100 μM), showing the Wnt-7b expression pattern in the less-differentiated tumor cells located at the tumor-stroma interface (arrowheads), and the keratinized, more-differentiated tumor cells located at the center of the tumor nests (asterisks**). **Right lower panel,** results of tumor Wnt-7b scoring for the 83 OSCC specimens.

**B.** Survival of the low-Wnt-7b and the high-Wnt-7b group of patients. **Upper panel,** Relapse-free survival; **lower panel,** Disease-specific survival.

**Figure 7. The effects of epigenetic modifiers on the re-expression 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. The black circle represents a methylated site, the open circle represents an unmethylated site.
B. RT-PCR analysis showing the expression of Meg-3 after treatment with the 5-Aza (5 µM) or TSA (100 nM).

C. RT-qPCR analysis showing the expression level of miRNAs after treatment with 5-Aza or TSA.

D. Methylation level of the CpG island in the meg-3 promoter region in OSCC tumors (T) versus adjacent normal tissue (N). Data are represented as mean±SD, ***p<0.001.

E. Meg-3 level in HOK cells after arecoline treatment using RT-PCR.

F. RT-qPCR analysis of indicated miRNAs in HOK cells after arecoline treatment.

G. A scheme showing the regulation of Wnt-7b by mi-329 and -410 during oral carcinogenesis. The 14q32.2 cluster of miRNAs is silencing by arecoline induced DNA methylation on Meg-3 DMR. Downregulation of miR-329 and miR-410 result in the expression of Wnt-7b protein. Wnt-7b serves as an important agonist of Wnt/β-catenin signaling and leads to the expression of downstream targets, such as c-Myc and cycline D1, and consequently promotes tumor proliferation and invasion in oral carcinogenesis.
Figure 1

A

B

C

D

E

F
Figure 2

A

\[
\text{Wnt-7b 3'}-\text{UTR} \\
\text{H. Sapiens (Human)}
\]

\[
\text{hsa-miR-329} \\
3' - uuuuccaaauugg\text{GUCCACACA} - 5' \\
5' - 1719 - \text{uggcccccaauua} - \text{GUGGUUGU} - \text{u} - \text{1994} - \text{gguuauacuua} - \text{GUGGUUGU} - \text{u} - 3'
\]

\[
\text{hsa-miR-410} \\
3' - \text{uguccgGuAGAC} - \text{ACAUAUAUA} - 5' \\
5' - 1719 - \text{uggcccccaauua} - \text{GUGGUUGU} - \text{u} - \text{1994} - \text{gguuauacuua} - \text{GUGGUUGU} - \text{u} - 3'
\]

B

Relative Expression of miRNAs (RT-qPCR)

C

RT-PCR

GAPDH

Wnt-7b

\[\alpha\text{-tubulin}\]

W.B.

D

Relative luciferase activity

E

PM

AM

Wnt-7b

\[\alpha\text{-tubulin}\]

F

Relative Expression Level (RT-qPCR)

\[\text{miR-329} \quad \text{miR-410}\]

G

\[r = -0.629, p=0.0003 \ (n=28)\]

\[r = -0.444, p=0.018 \ (n=28)\]
Downregulated miR-329 and miR-410 promote the proliferation and invasion of oral squamous cell carcinoma by targeting Wnt-7b

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