miRNA-491-5p and GIT1 Serve as Modulators and Biomarkers for Oral Squamous Cell Carcinoma Invasion and Metastasis


Abstract
MicroRNAs offer tools to identify and treat invasive cancers. Using highly invasive isogenic oral squamous cell carcinoma (OSCC) cells, established using in vitro and in vivo selection protocols from poorly invasive parental cell populations, we used microarray expression analysis to identify a relative and specific decrease in miR-491-5p in invasive cells. Lower expression of miR-491-5p correlated with poor overall survival of patients with OSCCs. miR-491-5p overexpression in invasive OSCC cells suppressed their migratory behavior in vitro and lung metastatic behavior in vivo. We defined the G-protein—coupled receptor kinase-interacting protein 1 (GIT1)—as a direct target gene for miR-491-5p control. GIT1 overexpression was sufficient to rescue miR-491-5p-mediated inhibition of migration/invasion and lung metastasis. Conversely, GIT1 silencing phenocopied the ability of miR-491-5p to inhibit migration/invasion and metastasis of OSCC cells. Mechanistic investigations indicated that miR-491-5p overexpression or GIT1 attenuation reduced focal adhesions, with a concurrent decrease in steady-state levels of paxillin, phospho-paxillin, phospho-FAK, EGF/EGFR-mediated extracellular signal–regulated kinase (ERK1/2) activation, and MMP2/9 levels and activities. In clinical specimens of OSCCs, GIT1 levels were elevated relative to paired normal tissues and were correlated with lymph node metastasis, with expression levels of miR-491-5p and GIT1 correlated inversely in OSCCs, where they informed tumor grade. Together, our findings identify a functional axis for OSCC invasion that suggests miR-491-5p and GIT1 as biomarkers for prognosis in this cancer. Cancer Res; 74(3); 751–64. ©2013 AACR.

Introduction
Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide and accounts for more than 95% of all head and neck cancer (1). The incidence of oral cancer in Taiwan increased by 30% in the last 5 years, mortality rate grew by 25% with 30 to 49 years old men having the highest rate. More than 44,000 patients annually require sustained medical treatment (2, 3). Unlike other types of solid tumors, the metastasis of OSCCs mostly involves local invasion and often is restricted to the head and neck area. Cervical lymph node metastasis is a critical prognostic factor for OSCCs, the patients without such metastasis usually have higher survival rates (4–6). Like other cancers, oral cancer metastasis requires an extensive remodeling and degradation of extracellular matrix (ECM), in part, via increased expression of matrix metalloproteinases (MMP; ref. 7). However, the molecular mechanism regulating the invasion and metastasis of OSCCs is still largely unclear.

MicroRNAs (miRNA) are an evolutionarily conserved group of small RNAs of 18 to 24 nucleotides that inhibit or stimulate gene expression. microRNAs have been known to play important roles in various cancer progression and metastasis (8, 9); however, study of their roles in OSCC metastasis is relatively scarce (10).

GIT1 is a multifunctional scaffold protein found to be associated with paxillin and capable of stimulating lamellipodia formation and spreading of cells (11–13). Although the GIT1/paxillin complex is known to play a role in regulating focal adhesion formation and cell migration (14), the precise role of GIT1 in metastasis of cancer especially in OSCCs is...
unclear. Focal adhesion kinase (FAK) is a ubiquitously expressed non–receptor protein tyrosine kinase (PTK). Previous studies have indicated that FAK functions as a positive regulator of tumor invasion and is overexpressed in various cancers, including breast, head and neck, and ovarian cancers (15, 16). FAK/paxillin interaction controls cell motility in part through extracellular signal–regulated kinase (ERK1/2) activation (17). The EGFR receptor (EGFR) is a ubiquitously expressed receptor tyrosine kinase that is often upregulated in head and neck cancer (18). Activation of EGFR signaling is known to promote cancer cell proliferation and metastasis (19, 20). A previous report showed that GIT1 linked EGFR-mediated activation of ERK1/2 in 293 cells (21). However, the role of EGFR/GIT1/FAK signaling in oral cancer is unknown.

In this study, we found that miR-491-5p was substantially downregulated in 5 highly invasive OSCC lines and was also widely attenuated in OSCCs compared with paired normal tissues. Our data show that miR–491–5p targets GIT1 to inhibit OSCC cell focal adhesion formation, invasion, and metastasis via regulation of FAK, paxillin, ERK1/2, and MMP2/9. Thus, this study suggests that miR-491-5p is a metastasis suppressor and that miR–491–5p and GIT1 are significant biomarkers for OSCC metastasis.

Materials and Methods

Tissue samples acquisition

All clinical samples were acquired via protocols approved by the respective Institutional Review Boards. Thirty-three oral normal and tumor tissue pairs were obtained from the Oral and Maxillofacial Surgery Unit, Chi Mei Medical Center, Tainan, Taiwan, and Department of Public Health and Environmental Medicine, School of Medicine, College of Medicine, Kaohsiung Medical University, Taiwan. Tumor and normal oral tissue samples from surgical resection were snap-frozen in liquid nitrogen or embedded in RNAlater. The clinical characteristics of these 33 N/T pairs are shown in Supplementary Table S1. Forty-eight oral cancer samples for cDNA microarray analysis (22) and 189 OSCC tissues array slides were obtained from Nuclear Medicine, Chang Gung Memorial Hospital at Linkou Taiwan. Commercial tissue array slides containing 134 OSCC tissues were purchased from US Biomax Inc.

Cell culture, DNA and RNA transfections, and stable cell line generation

Human oral cancer cell lines CGHNC9, SAS, SCC25, OECM-1, and OC-3 have been described (23). CGHNC9, OECM-1, and SAS cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) with 10% FBS (Biological Industries). SCC25 cells were grown in DMEM/HAM F12 (Invitrogen) supplemented with 10% FBS and hydrocortisone (0.4 mg/mL, Sigma-Aldrich). OC-3 cells were grown in 1:1 mixture of DMEM and KSFM (Invitrogen) with 10% FBS. All cells were incubated at 37°C in 5% CO2. Cells were transfected using the LipofectAMINE 2000 (Invitrogen) and LipofectAMINE RNAiMAX (Invitrogen). The miR–491–5p cell lines stably expressing pcDNA6.2-EmGFP-miR-491-5p or pcDNA-flag-GIT1 were established by transfection with the respective plasmids followed by selection with neomycin (Sigma-Aldrich).

Vectors, antibodies, and reagents

The miR–491–5p was constructed in pcDNA6.2-EmGFP. GIT1 coding sequence was cloned in pCMV-HA- and pcDNA3-flag. The luciferase-3'UTR-wt reporter or luciferase-3'UTR-mutant (mt) plasmids were prepared by inserting the GIT1-3’-UTR-wt carrying a putative miR-491-5p binding site or its mutant sequence into the pGML3-control plasmid (sequences are shown in Supplementary Table S2). N-terminally (1–676 amino acid) truncated FAK (dominant-negative FAK) and dominant-negative MEK (K97A mutant) were constructed in pcDNA3. Antibodies for Western blotting and immunohistochemistry (IHC) are mouse anti-GIT1 from BD Transduction Laboratories and Bethyl Laboratories, Inc. The primers (for plasmid construction), antibodies, and reagents used are described in the Supplementary Material and Table S2.

3’UTR reporter assays

pGL3-GIT1-3’UTR-wt or pGL3-GIT1-3’UTR-mt was co-transfected with pre-miR–491–5p or pre-anti-miR–491–5p into OSCC cells. Luciferase assay was conducted using an assay kit (Promega). Renilla luciferase was cotransfected as a control for normalization.

Reverse transcriptase PCR and quantitative reverse transcriptase PCR

Reverse transcriptase (RT)-PCR and quantitative RT (qRT)-PCR were used to detect the miR–491–5p and mRNA expression. We designed a stem-loop RT primer specifically hybridizing with miR–491–5p or RNU6B, respectively, the latter was used for normalization (24). The detailed conditions and primers used for mRNA expression are listed in the Supplementary Material and Table S2.

Cell chemotactic migration and invasion assay

Chemotactic migration and invasion ability of OSCC cells were assessed using the Falcon Cell Culture Inserts with or without Matrigel (BD Biosciences) coating as described (25). The detailed conditions are described in the Supplementary Material.

In vivo metastasis assays

CB17-SCID mice were used for injection of OSCC cells orthotopically at oral buccal mucosa or via tail vein and monitored for 21 to 42 days before sacrifice. Lung tissues were removed, fixed, paraffin-embedded, serially sectioned, and subjected to hematoxylin and eosin (H&E) staining. The detailed conditions are described in the Supplementary Material.

Focal adhesion assay and immunofluorescence microscopy

Immunostaining was conducted to detect the vinculin level and focal adhesions in C9-lung-JV2 cells. The detailed conditions are described in the Supplementary Material.
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Western blotting
Detailed procedure is described in the Supplementary Materials and Methods.

Paxillin degradation assays
C9-lung-IV2 cells were transfected with negative control (NC) or GIT1-siRNA-3 and incubated for 48 hours, and MG132 at 10 μmol/L was added to the cells for 8 hours. C9-lung-IV2 cells were transfected with negative control or GIT1-siRNA, and cycloheximide (Sigma-Aldrich) at 20 μg/mL was added to the cells for 0, 4, and 12 hours.

IHC and FISH
IHC was conducted to detect GIT1 expression from paraffin-embedded oral cancer specimens. The slides were stained with primary antibody using an automatic slide stainer BenchMark XT (Ventana Medical Systems). FISH was conducted using the tyramide signal amplification (TSA) technology and the 5’-biotin-labeled miR-491-5p probe or a negative control probe [25 (nmol/L)/L; GeneDireX, Inc.] as previously described (26). The IHC score of GIT1 and FISH score of miR-491-5p for each specimen was graded as follows: +, weak; ++, moderate; ++++, strong.

Gelatin zymography
Gelatin zymography was used to detect MMP2/9 activity in C9-lung-IV2 cells using conditions detailed in the Supplementary Material.

Statistical analysis
Survival data was analyzed using the Kaplan–Meier method. Differences between experimental groups were calculated with the Mann–Whitney U test. Differences with P < 0.05 are considered to be statistically significant. The linear correlation coefficient (Spearman’s ρ) was used to estimate the relationship between miR-491-5p and GIT1 levels in the oral tumor specimens.

Results
Establishment of isogenic pairs of high and low invasive OSCC lines
Recent evidence has shown that microRNAs play a role in regulating cancer metastasis (8). To identify relevant microRNAs associated with invasive phenotypes of oral cancer, we established isogenic pairs of high and low invasive oral cancer lines through in vitro and in vivo selections. Using Boyden chamber invasion assay (in vitro selection), the oral cancer cell lines OC-3, OECM-1, SAS, and SCC25 were selected 5 to 8 cycles to derive the highly invasive sublines OC-3-I5, OECM-1-I8, SAS-I5, and SCC25-I6 in which the number following I denotes the cycles of selection. OC-3 and CGHNC9 lines were selected 1 to 2 cycles by in vivo infection of the respective cells into tail vein of CB17-SCID mice (in vivo selection), followed by isolation of tumor cells grown from lymph node metastases or lung metastases to obtain OC-3-3-I5-lymph-node-IV1, OC-3-I5-lymph-node-IV2, OC-3-I5-IV1, OC-3-I5-IV2, and CGHNC9-lung-IV2 (C9-lung-IV2) where the number following IV denotes the cycle selection. After invasion selection, we tested those lines for their migratory and invasive ability by conducting Boyden chamber migration/invasion assays. As shown in Supplementary Fig. S1, the migratory and invasive ability of the selected invasive lines was dramatically increased.

Downregulation of miR-491-5p was found in the highly invasive OSCC cells and was correlated with poor survival of the OSCC patient
Using microRNA array, we attempted to identify microRNAs associated with the invasive phenotype of the 5 selected highly invasive OSCC lines (SAS-I5, OECM-1-I8, SCC25-I6, OC-3-I5-, and OC-3-I5-lung-IV2) compared with their corresponding parentals. A series of microRNAs with 2-fold differential expression between the low and high invasive isogenic lines were identified. The differentially expressed microRNAs of each isogenic pair as shown in each circle and the microRNA common among all 5 isogenic pairs are displayed by the Venn diagrams (Fig. 1A). The miR-491-5p has a greater than 2-fold lower expression in all 5 in vitro and in vivo selected invasive lines (Fig. 1A). The reduced expression of miR-491-5p was verified by RT-PCR, the in vivo selected invasive lines displayed a greater decrease of the microRNA compared with their parentals (Fig. 1B and C). Furthermore, we examined 33 pairs of oral normal and cancer tissues for the miR-491-5p expression. The miR-491-5p was underexpressed in 29 of 33 OSCC samples compared with their matched normal tissues (Fig. 1D).

Kaplan–Meier survival analysis of 189 cases showed that OSCC samples with low miR-491-5p expression correlated with a significantly poorer survival than those with high miR-491-5p expression (Fig. 1E, top). The miR-491-5p low expression also had a correlation with lymph node metastasis (Fig. 1E, bottom). The results suggest that miR-491-5p may play a role in suppressing OSCC lymph node metastasis and could serve as a prognostic marker for OSCCs.

miR-491-5p inhibits migration, invasion, and lung metastasis of OSCC cells
Next, we tested whether miR-491-5p was able to suppress migration and invasion of oral cancer cells by Boyden chamber assay. As expected, miR-491-5p significantly inhibited migration and invasion of OSCC cells, which could be partially or fully rescued by anti-miR-491-5p (Fig. 2A). To evaluate whether miR-491-5p inhibited cancer cell metastasis in vivo, we used 2 experimental metastasis models, namely, tail vein injection and orthotopic implantation of cells in SCID mice. In the tail vein injection model, expression of miR-491-5p resulted in greatly decreased targeting of the C9-lung-IV2 cells to lung 24 hours after injection (Supplementary Fig. S2). As shown in Fig. 2B, the percentage of lung tumor area in the miR-491-5p transiently transfected group was reduced to 45% of the control. Similarly, in the orthotopic model (Fig. 2C), stable overexpression of miR-491-5p significantly suppressed lung metastasis of C9-lung-IV2 OSCC cells. miR-491-5p resulted in fewer metastatic foci and small nodules in lung. These results indicate that miR-491-5p is able to potently inhibit migration, invasion, and metastasis of OSCC cells.
GIT1 is a direct target of miR-491-5p

To understand the mechanism of the miR-491-5p regulated OSCC cell invasion and metastasis, we attempted to identify its target genes by using 2 computational software, TargetScan and DIANA-microT. Those methods identified 17 candidate genes to be potential targets of miR-491-5p (Supplementary Table S3). We were interested in those involved in regulating focal adhesion complexes as they had been implicated in cancer cell invasion and metastasis (15, 27). Using Gene Ontology (GO) analysis, we found a putative target of miR-491-5p, GIT1, which belongs to the focal adhesion related gene family. To determine whether GIT1 is a direct target of miR-491-5p, we constructed Luc-GIT1-UTR-wild-type (wt) and its 3’UTR mutant (mt) plasmids. Luciferase reporter assays
showed that miR-491-5p suppressed more than 50% transcriptional activity of the Luc-GIT1-3’UTR-wt reporter compared with the control, whereas the GIT1-3’UTR-mt where the putative miR-491-5p binding site had been mutated was refractile to the inhibition (Fig. 3A). We also found that miR-491-5p significantly inhibited the GIT1 mRNA expression in CGHNC9 and SCC25 cells, which was rescued by anti-miR-491-5p. As expected, Western blotting showed that overexpression of miR-491-5p dramatically suppressed GIT1 protein expression in CGHNC9 and SCC25 cells and the inhibition was substantially reverted by anti-miR-491-5p (Fig. 3B). Consistently, the in vivo selected lines with lower miR-491-5p expression showed elevated GIT1 mRNA levels (Supplementary Fig. S3). Those results suggest that miR-491-5p directly targets GIT1, resulting in downregulation of its mRNA and protein.
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Suppression of GIT1 by siRNA inhibits OSCC cell migration, invasion, and metastasis

GIT1 is a multidomain scaffold protein that is usually found to be associated with focal adhesions and is able to promote cell motility (28). We found that GIT1 was upregulated in the highly invasive oral cancer lines compared with their parental lines (Supplementary Fig. S4). To assess whether GIT1 level correlated with metastasis, we analyzed cDNA microarray data of a series of 48 OSCC samples. We first determined the median GIT1 expression level from the samples and then divided them into 2 groups, that is, the higher or the lower expression than the median level. We found that the higher GIT1 expression appeared to be correlated with cases having higher number of positive lymph nodes involvement (Fig. 4A), as the increased level of GIT1 was significantly associated with the development of lymph node metastasis (Fig. 4A), as the increased level of GIT1 expression appeared to be correlated with cases having higher number of positive lymph nodes involvement (Fig. 4B). Next, we examined whether GIT1 could affect cell migration, invasion, and metastasis. Three sets of GIT1-siRNAs were tested for their inhibitory efficacy by Western blotting. GIT1-siRNA-3 had the highest knockdown effect (Fig. 4C, left) and was used in the subsequent experiments. Knockdown of GIT1 significantly impaired OSCC cell migration and invasion (Fig. 4C, right). GIT1 was able to rescue miR-491-5p-mediated inhibition of OSCC cell invasion (Fig. 4D). To further assess the role of GIT1 in regulating metastasis, we injected GIT1-siRNA expressing or the control plasmid-transfected C9-lung-IV2 cells into SCID mice via tail vein. Knockdown of GIT1 resulted in about 5-fold reduction in the lung metastasis (Fig. 4E).

Re-expression of GIT1 significantly reverses miR-491-5p-mediated suppression of invasion and metastasis of C9-lung-IV2 cells

Ectopic expression of GIT1 could partially rescue cell invasion inhibited by miR-491-5p in vitro (Fig. 4D). Using the tail vein injection assay, we also found that ectopic expression of GIT1 rescued miR-491-5p-mediated inhibition of lung metastasis about 3-folds (Fig. 4F). The above results support our hypothesis that miR-491-5p targets GIT1 to inhibit OSCC invasion and metastasis.

Inhibition of GIT1 by miR-491-5p enhances degradation of paxillin and impairs focal adhesion signaling in OSCC cells

Previous studies indicated that GIT1 interacted with focal adhesion–related proteins, including paxillin and FAK (29, 30). To investigate the detailed mechanism through which the miR-491-5p targeted GIT1 to inhibit cell migration and invasion, we first examined the effect on FAK and paxillin activation upon

Figure 3. Identification of GIT1 as the direct target of miR-491-5p. A, effect of miR-491-5p on GIT1 3′-UTR-wt (GIT1 3′-UTR-wt-luc) and GIT1 3′-UTR-mutant luciferase reporters (GIT1 3′-UTR-mut-luc). Top, GIT1 3′-UTR-wt sequence and the GIT1 3′-UTR-mt in which the sequence in bold was mutated to abolish the miR-491-5p binding. Bottom, luciferase assays showing decreased activity after cotransfection of GIT1 3′-UTR-wt with miR-491-5p in CGHNC9 (left) and SCC25 (right) OSCC cells. The GIT1 3′-UTR-mt reporter was not affected by miR-491-5p. B, expression of GIT1 mRNA in CGHNC9 and SCC25 cells was measured by qRT-PCR from RNAs extracted from cells transfected with miR-491-5p alone or together with anti-miR-491-5p (anti-491-5p). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. C, the expression of GIT1 protein was detected by Western blotting of total proteins from cells transfected with miR-491-5p alone or together with anti-miR-491-5p (anti-491-5p). β-Actin was used as an internal loading control. Histograms represent means ± SD from 3 independent experiments. *, P < 0.05.
Figure 4. GIT1 expression correlates with lymph node metastasis and knockdown of GIT1 inhibits OSCC cell migration, invasion, and metastasis. A and B, analysis of cDNA microarray data from 48 OSCC tumor samples revealed an association between high number of lymph nodes involvement and increased GIT1 expression, determined from the array analysis. Spearman’s $r$ analysis was used to evaluate the relationship of GIT1 expression and lymph node metastasis. C, left, Western blotting of GIT1 in CGHNC9 cells transfected with the GIT1-siRNA or negative control (NC). Right, a dramatic decrease in migration and invasion ability was observed in CGHNC9 cells transfected with GIT1-siRNA compared with the NC. D, left, Western blotting of GIT1 from CGHNC9 cells transfected with the GIT1 plasmid or vector control. Right, overexpression of GIT1 significantly rescued CGHNC9 cell invasiveness inhibited by miR-491-5p. E, lung metastasis following tail vein injection of C9-lung-IV2 cells transiently transfected with GIT1-siRNA was dramatically reduced. F, lung metastasis index shows that transient transfection of GIT1 plasmid into C9-lung-IV2 cells expressing miR-491-5p significantly rescued their lung metastasis generated by tail vein injection. All images show H&E staining of the lung metastases ($\times 200$). Histograms represent means $\pm$ SD from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$. 

miRNA-491-5p Suppresses OSCC Metastasis
GIT1 depletion in OSCC cells. We hypothesized that suppression of GIT1 could lead to inhibition of focal adhesion signaling and expression of molecules involved in cancer cell invasion. Indeed, suppression of GIT1 by miR-491-5p reduced the level of p-FAK, its interactive protein paxillin and phospho-paxillin (Fig. 5A) whereas the mRNA of paxillin remained unaffected (Supplementary Fig. S5). GIT1 has been shown to bind and regulate activation of paxillin through FAK (13, 30). As the paxillin mRNA was not affected by GIT1 knockdown, we checked whether the decreased paxillin was due to enhanced proteasome degradation. The level of paxillin in GIT1-depleted cells with or without addition of MG132 (a proteasome inhibitor) was compared. The result showed that treatment of MG132 reversed the effect of GIT1 depletion on paxillin level.

Figure 5. GIT1 depletion enhances degradation of paxillin and reduces FAK phosphorylation and decreases focal adhesion formation. A, Western blot analysis showing expression of miR-491-5p or knockdown of GIT1 decreased phosphorylation of paxillin, FAK, and reduced level of paxillin protein. B, knockdown of GIT1 significantly decreased paxillin stability in OSCC cells. The paxillin levels in control and GIT1-depleted C9-lung-IV2 cells treated with cycloheximide (20 µg/mL) for the indicated times show a faster decay in the GIT1 depleted cells. D, C9-lung-IV2 cells expressing the indicated plasmids were seeded in dishes coated with fibronectin for 120 minutes (top) and analyzed for total focal adhesion (FA) area per cell (bottom). The focal adhesions and stress fibers were revealed with anti-vinculin (green) and rhodamine (red), respectively. Histograms represent means ± SD from 3 independent experiments. * * P < 0.05.
(Fig. 5B), suggesting that knockdown of GIT1 increased paxillin degradation via proteasome pathway in oral cancer cells. To further confirm depletion of GIT1, resulting in decreased paxillin stability, we conducted protein degradation experiment in cells treated with cycloheximide (Cyclohex). Paxillin was stable over a course of 12 hours in the control cells. In contrast, paxillin stability was significantly decreased in the GIT1-depleted cells (Fig. 5C). We further tested whether GIT1 depletion could affect OSCC cell focal adhesion and lamellipodia formation. As shown in Fig. 5D, vinculin level and focal adhesions were significantly decreased in the miR-491-5p–transfected C9-lung-IV2 cells, which was reversed by overexpression of GIT1. Likewise, the focal adhesions were disrupted in the si-GIT1–transfected C9-lung-IV2 cells (Fig. 5D). These results suggest that miR-491-5p–mediated decrease of GIT1 results in reduced FAK activation and increased paxillin degradation, leading to reduced focal adhesion formation of OSCC cells.

**Dominant-negative form of FAK partially inhibited OSCC cell invasiveness that could be rescued by GIT1**

We further determined the role of FAK in miR-491-5p–targeted GIT1 downregulation and inhibition of migration and invasion. Our data showed that GIT1 promoted cell invasion could be reversed by dominant-negative FAK (DN-FAK; Supplementary Fig. S6B). Western blot analysis confirmed the downregulation of p-FAK by DN-FAK in C9-lung-IV2 cells overexpressing GIT1 (Supplementary Fig. S6A). Those data suggested that downregulation of GIT1 by miR-491-5p could suppress migration, invasion, and metastasis of OSCC cells through degradation of paxillin and reduced focal adhesion signaling, including FAK and ERK1/2 activation.

**miR-491-5p targets GIT1 to regulate expression level and activity of MMP2/9 via EGFR/ERK1/2 signaling pathway**

MMP2/9 have been implicated in tumor cell invasion and metastasis (31, 32). We observed that overexpression of miR-491-5p or knockdown of GIT1 decreased MMP2/9 mRNA (Fig. 6A) and activity (Fig. 6B) in OSCC cells. Previous studies indicated that the expression level and activity of MMP2/9 were regulated by ERK1/2 in HEK293 and monocytic cells (33, 34). Our data also showed that the expression level of MMP2/9 was reduced by PD98059 (an MEK inhibitor) in OSCC cells (Supplementary Fig. S7). GIT1 was shown to link the EGFR to ERK1/2 activation in HEK293 cells (21). However, the role of GIT1 in the regulation of MMPs in oral cancer cells is unknown. To check whether the miR-491-5p–GIT1 pathway could regulate ERK1/2 signaling, which in turn affected MMP2/9 expression, we examined the effect of miR-491-5p and GIT1 on EGF-induced ERK1/2 activation. CGHNC9 cells were transfected with control-siRNA, miR-491-5p, or GIT1-siRNA, serum starved for 24 hours, and then stimulated with EGF. In the control cells, EGF rapidly stimulated robust activation of ERK1/2. In contrast, miR-491-5p or GIT1-siRNA–treated cells showed significantly reduced activation of ERK1/2 (Fig. 6C). Thus, reduced GIT1 expression is associated with dramatic inhibition of EGF-stimulated ERK1/2 activation. Consistently, overexpression of miR-491-5p resulted in significantly inhibition of the growth beyond 72 hours especially upon EGF stimulation (Supplementary Fig. S8B). In addition, our data showed that GIT1 increased MMP2/9 mRNA expression could be reduced by dominant-negative MEK or PD98059 (Supplementary Fig. S9B). Western blot analysis confirmed the downregulation of p-ERK1/2 by DN-MEK and PD98059 in C9-lung-IV2 cells overexpressing GIT1 (Supplementary Fig. S9A). These results suggest that miR-491-5p targets GIT1 to suppress MMP2/9 expression and activity, and this is likely to be due to inhibition of ERK1/2 activation in OSCC cells.

**Correlation of miR-491-5p and GIT1 expression with grades of OSCCs and inverse correlation between miR-491-5p and GIT1**

There have been no studies reporting the role of GIT1 in oral cancer so far. We examined GIT1 expression in the matched normal and cancerous oral tissue specimens to assess its clinical connection. We found that in 26 of 33 cases of OSCC samples, the GIT1 level was 1.5- to 23-fold higher compared with their matched normal tissues (Fig. 7A), and the GIT1 level was inversely correlated with miR-491-5p expression (Fig. 7B). Furthermore, 189 OSCC samples showed that 51 patients with grade 1 had longer survival time than 138 patients with grade 2 and 3 (Fig. 7C). We next determined whether tumor grade was correlated with GIT1 expression by IHC staining of oral cancer tissue arrays. For each section, staining was measured as weak (+), moderate (++), and strong (+++). We observed that GIT1 level was higher in the moderately (grade 2) and poorly differentiated (grade 3) than in the well differentiated tumors (grade 1; Fig. 7D). About 79% of cases with grade 2 to 3 had moderate-to-strong expression of GIT1, whereas 79% of cases with grade 1 had weak expression of GIT1 (Fig. 7D, table). Thus the GIT1 level appeared to correlate with the aggressiveness of oral cancer. Furthermore, miR-491-5p signals were relatively weak in moderately and poorly differentiated tumor samples (Fig. 7D). Seventy-seven percent of cases with grade 2 to 3 had weak-to-moderate expression of GIT1, whereas 79% of cases with grade 1 had weak expression of GIT1 (Fig. 7D, table). Thus the GIT1 level appeared to correlate with the aggressiveness of oral cancer.

**Discussion**

OSCC has a high incidence of neck metastasis and often metastasizes contralaterally (35). It is widely known that the lymph node metastases is an important prognosticator predicting survival of patients with OSCCs (36–38). Despite accumulating evidences pointing to the regulatory role of microRNAs in cancer progression and invasion, its regulatory role in oral cancer metastasis is still poorly understood. This study aimed to identify relevant microRNAs and their downstream target genes involved in the regulation of cancer metastasis in OSCCs. For the first time, we establish the miR-491-5p–GIT1-FAK-paxillin and miR-491-5p–GIT1–ERK1/2 pathways in the
regulation of OSCC cell invasion and metastasis. We also show that low expression of miR-491-5p and high expression of GIT1 correlate with aggressiveness and lymph node metastasis of OSCC. Furthermore, we showed that the level of miR-491-5p is significantly correlated with overall survival of OSCCs. Interestingly, in a separate study, we found that GIT1 also played an important role in lymph node metastasis of breast cancer (Chan and colleagues, unpublished).

We found that miR-491-5p level decreased significantly in the highly invasive OSCC lines compared with their corresponding less invasive parental cells and that miR-491-5p suppressed migration, invasion, and metastasis of oral cancer cells mainly via targeting GIT1 to affect FAK and ERK1/2 signaling. A recent study reported the inhibitory effect of miR-491-5p on expression of MMP9 and invasion of glioblastoma multiforme cells (39). Our data also show that MMP9 mRNA expression level and activity are decreased in the OSCC cells overexpressing miR-491-5p most likely via inhibition of ERK1/2 activation (Fig. 6). In another study, it was shown that overexpression of CD44 3'UTR interacted with and trapped endogenous miR-491-5p, leading to increased levels of fibronectin and collagen, resulting in enhanced cell motility and invasion in human breast cancer cells (40). Our finding in OSCC is in agreement with those studies suggesting miR-491-5p functions as an invasion suppressor in glioma cells and breast cancer cells. However, we show here that miR-491-5p inhibits

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**Figure 6.** Effect of GIT1 on activation and expression of MMP2/9 and on EGF-induced ERK1/2 phosphorylation. A, relative MMP2/9 mRNA levels in CGHNC9 cells transfected with the indicated plasmids. B, top, Western blotting of GIT1 expression in CGHNC9 cells transfected with miR-491-5p, GIT1-siRNA, or negative control (NC). Bottom, CGHNC9 cells were preincubated in serum-free medium for 24 hours, and conditioned media were extracted and analyzed by gelatin zymography. C, CGHNC9 cells were transfected with the indicated plasmids for 8 hours, serum-starved for 24 hours, and then treated with 10 ng/mL EGF for the indicated times. Histograms represent means ± SD from 3 independent experiments. *P < 0.05, **P < 0.01.
OSCC invasion and metastasis via targeting GIT1, which is distinct from those 2 studies. As shown in Supplementary Fig. S8A, miR-491-5p although had no significant effect on proliferation of OSCC cells before 72-hour time point did affect the growth beyond that time point especially upon EGF stimulation likely due to inhibition of ERK1/2 activation (Supplementary Fig. S8B). Our data also showed that expression of miR-491-5p in C9-lung-IV2 cells resulted in a significant decrease of lung metastasis compared with the control in SCID mice at 24-hour time point (Supplementary Fig. S2). It appears that...
miR-491-5p targets and regulates distinct pathways to manifest its biologic function in different types of cancer cells. Our study identified miR-491-5p as a novel metastasis suppressor in oral cancer.

The differential expression level of miR–491-5p and difference in migration/invasion ability is difficult to equate quantitatively. We think at least there are 3 possible explanations; first, a given miRNA could have multiple targets involved in regulating migration/invasion; second, a 2-fold difference in miRNA could lead to a greater decrease in the target, resulting in more than 2-fold difference in migration/invasion; and third, the same effector gene could be targeted by miRNAs other than miR–491-5p.

As a scaffold protein associated with integrin, GIT1 plays a functional role in cell migration (11, 12), focal adhesion formation (28), and angiogenesis (41). For example, GIT1 promotes migration in A431 cells by interacting with the MYO18A-PAK2-8pix complex (11). GIT1 is also an important regulator for VEGF-induced endothelial cell podosome formation (41). Moreover, several previous studies showed that GIT1 was associated with p-ERK1/2 in focal adhesions after EGF stimulation in HeLa cells (42, 43) and affected focal adhesion formation in osteoblasts following platelet-derived growth factor stimulation (28). Although GIT1 has been implicated in the regulation of cell motility, its role in invasion and metastasis of OSCC cells has not been reported before. For the first time, our study indicates that GIT1 plays an important role in invasion and metastasis of oral cancer cells and it is targeted and downregulated by miR–491-5p in the highly invasive and poorly differentiated tumor cells.

Regulation of focal adhesion complexes involving FAK and paxillin are known to be important in cell invasion (44). Our data show that knockdown of GIT1 led to decreased FAK activity (Fig. 5A) and increase of proteasome-mediated degradation of paxillin (Fig. 5B and C). Together, these findings suggest that miR–491-5p inhibits invasion and metastasis of OSCC cells through depletion of GIT1, leading to disruption of focal adhesion complexes. Although GIT1 is often implicated in the regulation of cell focal adhesion, information about its role and regulatory mechanism for the invasion and metastasis in oral cancer has not been explored previously. Interaction between FAK and paxillin plays an important role in focal adhesion signaling and regulation of cell motility (45), in part, via ERK1/2 activation (17). Furthermore, a previous study indicated that paxillin/−/− MEF cells showed several phenotypic changes, including reduced cell migration (46). In addition, paxillin/−/− MEF still have focal adhesion (with abnormal shapes). Our data also showed that GIT1-depleted OSCC cells had decreased level and activity of paxillin protein and reduced cell migration. Depletion of FAK inhibited the phosphorylation of paxillin as expected as paxillin is a substrate of FAK and knockdown of paxillin also inhibited phosphorylation of FAK (Supplementary Fig. S10). These results suggested that depletion of GIT1 reduced paxillin stability, leading to decreased FAK phosphorylation in agreement with the report of Hagel and colleagues (46). Therefore, the loss of focal adhesions upon miR–491-5p overexpression or knockdown of GIT1 may not be attributed to the reduction of paxillin, but mainly to the reduction of GIT1. Among the potential upstream activation of ERK1/2 is EGF, which has been previously implicated in oral cancer development and progression (47, 48). Our data show that depletion of GIT1 significantly reduces EGF-induced ERK1/2 activation in OSCC cells (Fig. 6C). This is in agreement with a previous study indicating that GIT1 links the EGF to ERK1/2 activation by associating with MEK1 (21). Thus, we propose a regulatory signaling pathway of OSCC metastasis consisting of miR–491-5p and its downstream target, GIT1, which functions as a scaffold for focal adhesion signaling and EGF-induced ERK1/2 activation.

We showed that transient transfection with miR–491-5p or knockdown of GIT1 dramatically inhibited MMP2/9 mRNA expression and activity (Fig. 6A and B). We also showed that treatment of dominant-negative MEK or PD98059 (Supplementary Fig. S7) reduced the MMP2/9 expression level. It was shown that ERK1/2 pathway enhanced the invasion of lung cancer cells by increasing MMP2/9 expression and activity (33). Thus, the inhibition of MMP2/9 by miR–491-5p and knockdown of GIT1 is likely, in part, due to decreased ERK1/2 activation in OSCC cells.

Our analysis of clinical samples revealed a low miR–491-5p and high GIT1 expression pattern in the advanced OSCC and there appeared to exist an inverse relationship between miR–491-5p and GIT1 expression. The miR–491-5p seems to have no effect on OSCC cell growth. Thus, miR–491-5p expression is less likely to be involved in tumor initiation or growth; rather, it is more likely to be involved in the invasive progression when it needs to be downregulated to allow overexpression of GIT1 for cancer cell motility and MMP production/activation. It would be interesting to explore the upstream regulator for miR–491-5p downregulation during the metastatic progression of OSCCs.

In conclusion, our study has identified miR–491-5p as a novel metastatic suppressor through regulation of its downstream target, GIT1, resulting in perturbation of FAK/paxillin and ERK1/2 signaling as well as MMP expression and activity in oral cancer cells (Fig. 7E). The miR–491-5p and GIT1 could potentially serve as metastatic prognosis biomarkers and targets for intervention of OSCC metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Cancer Res; 74(3) February 1, 2014

Cancer Research

Published OnlineFirst December 12, 2013; DOI: 10.1158/0008-5472.CAN-13-1297

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Secured grant support for the study and acquisition of clinical specimens: L.-H. Wang

Acknowledgments

The authors thank the Pathology and Microarray Core Laboratories of the National Health Research Institutes for H&E and IHC staining and microRNA array analysis, respectively. They also thank Hsin Lei Yao for discussion.

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doi:10.1158/0008-5472.CAN-13-1297

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