Original Research

Long non-coding RNA TIRY promotes tumor metastasis by enhancing epithelial-to-mesenchymal transition in oral cancer

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Impact statement

This study demonstrated the novel lncRNA, TIRY, enhances epithelial-to-mesenchymal transition in cancer-associated fibroblasts and promotes the metastasis of tumor via miR-14 sponging in oral squamous cell carcinoma, and thus provide a novel molecular mechanism underlying the role of TIRY in CAFs in tumor biology and a potential target in OSCC. Further, the data showed that TIRY expression was negatively correlated with miR-14 transcription levels and was associated with poor prognosis in OSCC specimens. Therefore, TIRY may be a potential prognostic biomarker of overall survival and progression-free survival in OSCC. Moreover, TIRY adds to the understanding of regulatory mechanisms involved in CAFs and epithelial cancer cells in OSCC and may provide novel insights for further understanding tumor biology.

Abstract

Long non-coding RNAs (lncRNAs) modulate a variety of cancerous biological processes, including the promotion of tumorigenicity in tumor parenchymal cells. However, there is a lack of studies assessing the regulation of lncRNAs in cancer-associated fibroblasts. In the present study, a novel lncRNA, TIRY, was found to act as a miRNA sponge and to down-regulate miR-14 expression in oral squamous cell carcinoma (OSCC). Fluorescence *in situ* hybridization assay was used to evaluate TIRY expression in OSCC tissues. Survival analysis in a prospective cohort revealed a correlation between high TIRY expression and short progression-free survival. Subsequently, TIRY expression in cancer-associated fibroblasts and primary fibroblasts from adjacent normal (para-carcinoma) tissues was assessed using quantitative reverse transcription polymerase chain reaction. TIRY overexpression in cancer-associated fibroblasts isolated from OSCC tissues was induced by overexpressing the TIRY plasmid, and candidate microRNA expressions were assessed using quantitative real-time polymerase chain reaction. Moreover, the expression of proteins related to epithelial-to-mesenchymal transition (EMT) was determined; the proliferation, metastasis, and invasion of cancer cells co-cultured with TIRY-overexpressing cancer-associated fibroblasts were determined. We found significantly decreased miR-14 expression in cancer-associated fibroblast-derived exosomes and increased expression of EMT markers including transcription factors (Snail and FOXC2) and cellular scaffolding proteins (α-SMA, β-catenin, and FSP1). TIRY overexpression in cancer-associated fibroblasts activated the Wnt/β-catenin signaling pathway and promoted the invasion and metastasis of OSCC cells through miR-14 sponging based on cancer-associated exosome secretion. Our findings provide a novel molecular mechanism underlying the role of TIRY in cancer-associated fibroblasts in tumor biology; moreover, TIRY is a potential therapeutic target in OSCC.

Keywords: Long non-coding RNAs, epithelial-to-mesenchymal transition, cancer-associated fibroblasts, metastasis, miR-14, oral squamous cell carcinoma

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Introduction

Recently, the prevalence of oral squamous cell carcinoma (OSCC) is continuously and gradually increasing worldwide, with approximately 3,00,000 cases occurring every year.[1,2] Despite multiple advancements in the diagnosis and treatment of OSCC, approximately half of all patients with OSCC are still at a high risk of mortality within five-years of diagnosis even after receiving standard treatment.[3] Long-term prognosis in such patients is complicated owing

to the manifestations of advanced cancer and multifocal diseases of the upper digestive tract.[3]

The mechanism underlying the transformation of normal squamous oral mucosa (OM) to premalignant and malignant tissues is still unclear. Cancer-associated fibroblasts (CAFs) are one of the most abundant cell components in tumor tissues, and evidence has demonstrated their role in promoting carcinogenesis and cancer progression in different cancers, including liver cancer and ovarian cancer.[4–6] Recently, studies have reported on CAFs in OSCC. A previous study has demonstrated that human primary osteoblasts cultivated in OSCC- and CAF-conditioned media displayed an obvious increase in RANKL level and a decrease in OPG mRNA expression.[7] RANKL release was dramatically enhanced in primary oral fibroblasts that may differentiate into a CAF-like phenotype. Cultivating murine macrophages in a CAF-conditioned medium (isolated from OSCCs) promoted osteoclast formation. The aforementioned study was aimed at elucidating the function of CAFs in the bone metastasis of OSCC.[7] Another study revealed that CAFs induced more aggressiveness in cancer cells relative to normal fibroblasts. Compared with injecting tumor cells alone or injecting a co-culture of OSCC cells and normal fibroblasts, injecting a co-culture of OSCC cells and CAFs resulted in earlier tumor formation and greater tumor volume along with increased angiogenesis and lymphangiogenesis.[8] However, the mechanisms underlying the function of CAFs in the invasion and metastasis of epithelial-derived carcinomas such as OSCC are still unclear.

Long non-coding RNA (lncRNA) is a type of RNA that does not encode proteins and is composed of >200 nucleotides; such RNAs are known to play a role in various pathological and physiological processes.[9,10] Recently, the dysregulation of lncRNA expression has been reported in different cancer types and has been correlated with cancer aggressiveness. Reportedly, lncRNAs can be delivered to cells via CAF-derived exosomes and influence epithelial cancer cells.[11] Numerous studies have shown that cancer cells, CAFs, and NFs can influence each other by producing exosomal miRNAs. miRNA dysregulation influences the formation of CAFs and leads to effects such as drug resistance and poor prognosis.

Apart from the dysregulation of lncRNAs, an aberrant increase or a pathologic deficiency of miRNAs as well as exosomal miRNA transfer are associated with an aggressive phenotype of cancer cells such as invasion, metastasis, and chemoresistance.[6] A study showed that CAF exosomes promoted the differentiation of cancer stem cells, significantly enhancing chemoresistance via modulation of miR-21 and miR-146a. Additionally, CAFs pre-treated with gemcitabine demonstrated increased drug resistance, possibly due to an increase in miR-146a level in the exosomes, indicating that drug-induced exosome miRNAs are closely correlated with chemotherapeutic drug resistance.[12] Moreover, Josson *et al.*[13] demonstrated that miR-409 upregulation leads to miR-409 release via extracellular vesicles, tumor induction, and EMT. Moreover, miR-320a is dramatically decreased in patients with HCC. It is also linked to PBX3 and thus inhibits HCC. The miR-320a–PBX3 pathway can suppress the MAPK pathway, which induces EMT and promotes MMP2 and CDK2 expressions.[14]

We found that insufficient miR-14 expression in exosomes derived from CAFs isolated from oral cancer tissues because of the aberrant expression of the lncRNA TIRY. Decreased miR-14 production in CAFs and CAF-derived exosomes leads to the activation of the Wnt/β-catenin signaling pathway and increase in epithelial-to-mesenchymal transition (EMT), enhancing the invasion and metastasis of OSCC. Further, TIRY expression measured using fluorescence *in situ* hybridization (FISH) assays may predict prognosis of patients with OSCC. Thus, TIRY may be a potential prognostic biomarker of overall survival and progression-free survival in OSCC. Moreover, TIRY adds to the understanding of regulatory mechanisms involved in CAFs and epithelial cancer cells in OSCC.

Material and methods

Patients and tissue specimens

A total of 145 patients with OSCC were enrolled in the study. All patients underwent surgery and primary tumor excision in combination with neck dissection. None of them received chemotherapy or radiotherapy before surgery. All diagnoses were conducted in accordance with the WHO Tumor Classification system. Complete follow-up data were obtained from the electronic medical record system of Qingdao Stomatology Hospital. The adjacent normal OM (para-carcinoma) tissues of patients who underwent primary tumor excision were used as control tissues. This study was approved by Qingdao Stomatology Hospital, and all patients provided informed consent by signing on the relevant form.

FISH assay

TIRY and miR-14 expressions were assessed in 145 OSCC samples using FISH assay with digoxin-labeled probes. Aperio ImageScope V11 was used to assess TIRY and miR-14 stainings and expressions, with a positive value indicating circMTO1 expression. The sequence of the circMTO1 probe was as follows; digoxin-5′ GCT TGC ACT TTC GCA TCG CCA TGA 3′-digoxin. For use in FISH assays, the TIRY probe had a sequence of 5′-TCA ACG CGT AGC TGC GTT CCA GTC AAC GTC CGT TGA ACG GGT-3′ and the miR-14 probe had a sequence of 5′-GTC TTC GAG CTA CCG GTA ATC TGC GCT-3′.

Cell culture

The TCA8113 cell line was procured from the Chinese Academy of Sciences and was cultured in DMEM with 10% FBS and 1% streptomycin–penicillin in a humid atmosphere with 5% CO_2 at 37°C. The medium was replaced every third day.

Isolation, identification, and culture of NFs and CAFs

Fresh and aseptic tissues were cultivated in HBSS solution containing streptomycin–penicillin (200 μg/mL). After washing thrice with sterile PBS, adipose and epithelial

tissues were excised and the connective tissue was cut into small pieces, which were cultivated in DMEM with 20% FBS, 0.25% trypsin, penicillin (100 U/ml), glutamine (20 μg/ml), and streptomycin (100 μg/ml) with 5% CO_2 at 37°C. Tissues were observed on a daily basis, and the medium replaced after one day for the first time and subsequently every three days. Curettage in combination with trypsinization was used for purifying fibroblasts. Cultures at the third stage were used for identifying NFs and CAFs. An anti-pan-cytokeratin antibody (1:200) was used to identify epithelial cells, and anti-vimentin (1:200) and anti-α-smooth muscle actin antibodies (1:200) to identify CAFs.

Data analysis from lncRNA RNA-seq datasets

The genome-wide profiling of lncRNA expression in normal OM, oral submucous fibrosis (OSF), and OSCC tissues performed via RNA sequencing has been previously reported in GEO DataSets (GSE125866). Bioinformatics methods were applied for differential lncRNA and visualization analyses using the R software package (GGPLOT2). Quantitative RT-PCR was used to verify the identified lncRNAs.

Transfection

Cells were transfected using Lipofectamine 2000. Briefly, after dilution, adenoviral vectors or siRNAs were incubated for 5 min and RNA/DNA-Lipofectamine 2000 was added. The resulting solution was incubated for another 20 min and then used for cell transfection, which was followed by cell incubation for two days. siRNAs and adenoviral vectors were procured from JIKAI Bio Tech Ltd. (Shanghai, China).

qRT-PCR

Total RNA was isolated using the TRIzol reagent, and cDNA was prepared using the PrimeScript RT Reagent Kit. Briefly, qRT-PCR was conducted using a sequence comprising 40 cycles at 95°C for 5 min, 95°C for 15 s, 58°C for 30 s, and 74°C for 30 s. The primers used were as follows: TIRY, F: GTGTCAGCATAGCCCATACAGTAAA, R: CAGTCACATGCTCACACACAAG; Snail, F: ACATCAGCCTGGTGAACAAAA, R: CATTAACACATGCGACACCAA; miR-14, F: GATCAGCATCACACCTGCC, R: GCATGACCACTCTGGCAAAGG; and GAPDH, F: TCACACCTCGGCATAAGGGGAGTAGA, R: ATCCGTGGACACCCATGGAGG.

Western blotting

Proteins were isolated from cells using RIPA buffer and were separated using electrophoresis. These proteins were then transblotted onto a PVDF membrane (300 mA, 100 min), which was subsequently blocked with 5% skim-milk for 120 min and incubated with primary antibodies overnight at 4°C [anti-Snail (1:1000), anti-β-catenin (1:500), anti-Wnt3A (1:1500), anti-phosphorylated β-catenin (1:500), anti-ZEB1 (1:1000), anti-α-SMA (1:2000), or anti-GAPDH (1:2000)] and then with secondary antibodies for 120 min. Bands developed using ECL reagents were visualized using an imaging software.

Cell migration and invasion assays

Cells were suspended in DMEM medium without serum (1.0×10^5/mL). Transwell chambers were inserted in 24-well plates. Basolateral and apical chambers contained medium with 10% FBS (0.5 mL) and the aforementioned cell suspension (0.2 mL), respectively. After two days, cells were fixed with 5% POM and stained with 0.1% gentian violet and were enumerated (20×). Transwell chambers that were pre-coated with matrigel were used in the cell invasion assays.

RNA pull-down assay

TIRY, TIRY-mut, or beads were procured from JIKAI Bio Tech Ltd. The assay was performed in the HEK-293 and primary CAFs and using a MEGAscript T7 Transcription Kit according to the manufacturer's instructions. RNAs pulled down using the beads were detected using qRT-PCR.

Dual-luciferase reporter assay

Cells were transfected with miR-14 and pMiR-reporter vector-Wnt3A 3′UTR and were cultured for two days and then lysed to determine luciferase activity using DLRA. Vectors were obtained from GenePharma. All the experiments were conducted three times.

Conditioned medium preparation and exosome extraction

CAF-derived exosomes were extracted using the ExoQuick-TC Exosome precipitation solution (System Biosciences, Mountain View, CA, USA) as previously described.[12] The precipitated exosome pellet was resuspended in DMEM medium and incubated for 72 h with recipient cells.

Medium transfer

CAFs (donor) and TCA8113 (recipient) cells were inoculated in six-well plates (6×10^4/well), the donor medium was centrifuged (1200 r/min for 5 min), and the supernatant was added to the recipient cells for six successive days. CAFs and TCA8113 cells were inoculated in exosome-free and conditioned medium (1×10^6/flask), respectively, followed by centrifugation to remove exosomes and retain proteins. Cells were cultivated in conditioned medium for four days. The medium was replaced once a day, and the number of live cells was determined daily.

Statistical analysis

The independent samples Student's *t*-test was used to analyze the difference between the two groups. Linear regression analysis was used to identify the correlation between continuous variables. Survival curves were generated using the Kaplan–Meier method, and differences between the groups were analyzed using the log-rank test. Area

under the curve (AUC) was determined based on diagnostic evaluation tests and receiver operating characteristic curves. $P < 0.05$ indicated a significant difference; all P values were double-tailed. All statistical analyses were conducted using the SPSS software.

Results

Increased TIRY expression and decreased miR-14 expression were noted in OSCC tissues

We analyzed raw data from GEO DataSets (GSE125866) focusing on the lncRNA expression profile associated with the malignant progression of OSF. We selected eight para-carcinoma tissue samples and carcinoma tissue samples from eight patients with OSCC. And we eventually identified 44 lncRNAs (upregulated, 29; downregulated, 17) in OSCC tissues with significantly different expression compared with those in normal tissues (Figure 1(a)). We then focused on the five most upregulated lncRNAs in OSCC tissues and measured their expressions using quantitative RT-PCR (Figure 1(b)). We found that TIRY was significantly and highly expressed in OSCC tissues compared with that in para-carcinoma tissues (Figure 1(b)). We also found that TIRY expression was significantly higher in primary fibroblasts from OSCC tissues than in those from para-carcinoma tissues (Figure 1(c)). Next, we measured the expression of 10 candidate microRNAs with high context scores, which may be sponged and downregulated as determined using bioinformatics analysis using Target Scan. We found that miR-14 expression was significantly higher in primary fibroblasts from OSCC tissues than in those from para-carcinoma tissues (Figure 1(d) and Supplementary Figure 1). In *in-vitro* experiments, RNA pull-down assays also confirmed that TIRY endogenously binds to miR-14 in the HEK-293 cell line and primary CAFs (Figure 1(e) and Supplementary Figure 2(a)). Further, qRT-PCR revealed that inhibition of TIRY by small interference RNA would lead to the increased expression of miR-14 in the primary CAFs and TIRY overexpression negatively regulated miR-14 expression in HEK-293 cell line (Figure 1(f) and Supplementary Figure 2(b)). These data demonstrated that increased TIRY expression in OSCC tissues and CAFs negatively regulates miR-14 expression in these tissues.

Higher TIRY expression in OSCC tissues predicted poor prognosis

We constructed a prospective cohort of patients with OSCC (154 patients) and performed a three-year follow-up. The clinical baseline characteristics and demographic data are shown in Table 1. There is no significant difference in the clinical baseline characteristics and demographic data shown in Table 1 between the patients with a higher or lower expression of IncRNA TIRY. TIRY and miR14 expressions in all 154 OSCC tissues and paired para-carcinoma tissues were examined using FISH assays. Results indicated that the ratio of TIRY expression in OSCC tissues to that in para-carcinoma tissues was negatively correlated with the corresponding miR-14 expression ratio (Figure 2(a)). TIRY in OSCC tissues was quantified using FISH assay by measuring the relative fluorescence intensity. Receiver operating characteristic curves and diagnostic evaluation tests demonstrated the potential diagnostic value of TIRY expression in distinguishing patients with a higher risk of recurrence or metastasis within one year of diagnosis from those with a lower risk (AUC = 0.897, Figure 2(b)). However, the predictive value of the three-year mortality of patients was relatively small because of low AUC value (AUC = 0.576) (Figure 2(c)). Based on the best cut-off value of the relative fluorescence intensity of TIRY measured using FISH assays, patients were classified into high-expression or low-expression groups. One-year progression-free survival three-year overall survive in the high-expression group were lower than those in the low-expression group (Figure 2(d) and (e)). The odds ratio is 1.81 (95% confidence interval 1.56, 3.19) for the one-year progression-free survive in higher expression group compared to low expression group. The odds ratio is 1.33 (95% confidence interval 1.12, 2.39) for the one-year progression-free survive in higher expression group compared to low expression group.

TIRY promoted EMT in CAFs and enhanced invasion and metastasis in TCA8113 cells

To investigate the role of TIRY expression in CAFs, we evaluated EMT marker protein expressions. We found that TIRY overexpression significantly increased EMT-related transcription factor (Snail and ZEB1) and cellular scaffolding protein (α-SMA and β-catenin) expressions in CAFs isolated from OSCC tissues (Figure 3(a) and (b)). Considering the crucial role of external factors in cell growth and survival, we explored whether factors produced by CAFs could influence biological processes in epithelial cancer cells. We investigated the influence of CAF-conditioned medium on the proliferation of TCA8113 cells. An equivalent number of primary CAFs with or without TIRY overexpression were cultivated in DMEM for one day. CAF-conditioned medium was then added to TCA8113 cells for six days. CAF-conditioned medium from primary TIRY-overexpressing CAFs had no effect on the proliferation of TCA8113 cells (Figure 3(c)). Subsequently, we assessed if CAF-conditioned medium from primary TIRY-overexpressing CAFs could promote the invasion and metastasis of TCA8113 cells. We found that this medium led to a significant increase in the invasion and metastasis of TCA8113 cells as assessed using the transwell assay and wound healing test (Figure 3(d) and (e)).

TIRY overexpression decreased miR-14 and snail expressions in CAF-derived exosomes

As CAF-conditioned medium increased the invasion and metastasis of TCA8113 cells, we explored whether miR-14 could be transferred to these cells through CAF exosomes. We first measured miR-14 and Snail expressions in TIRY-overexpressing CAFs and then explored whether Snail mRNA or miR-146a expression was upregulated in CAF exosomes. Using qRT-PCR, we found that TIRY overexpression led to an increase in Snail expression and a decrease in miR-1 expression in CAFs (Figure 4(a) and (b)); we also

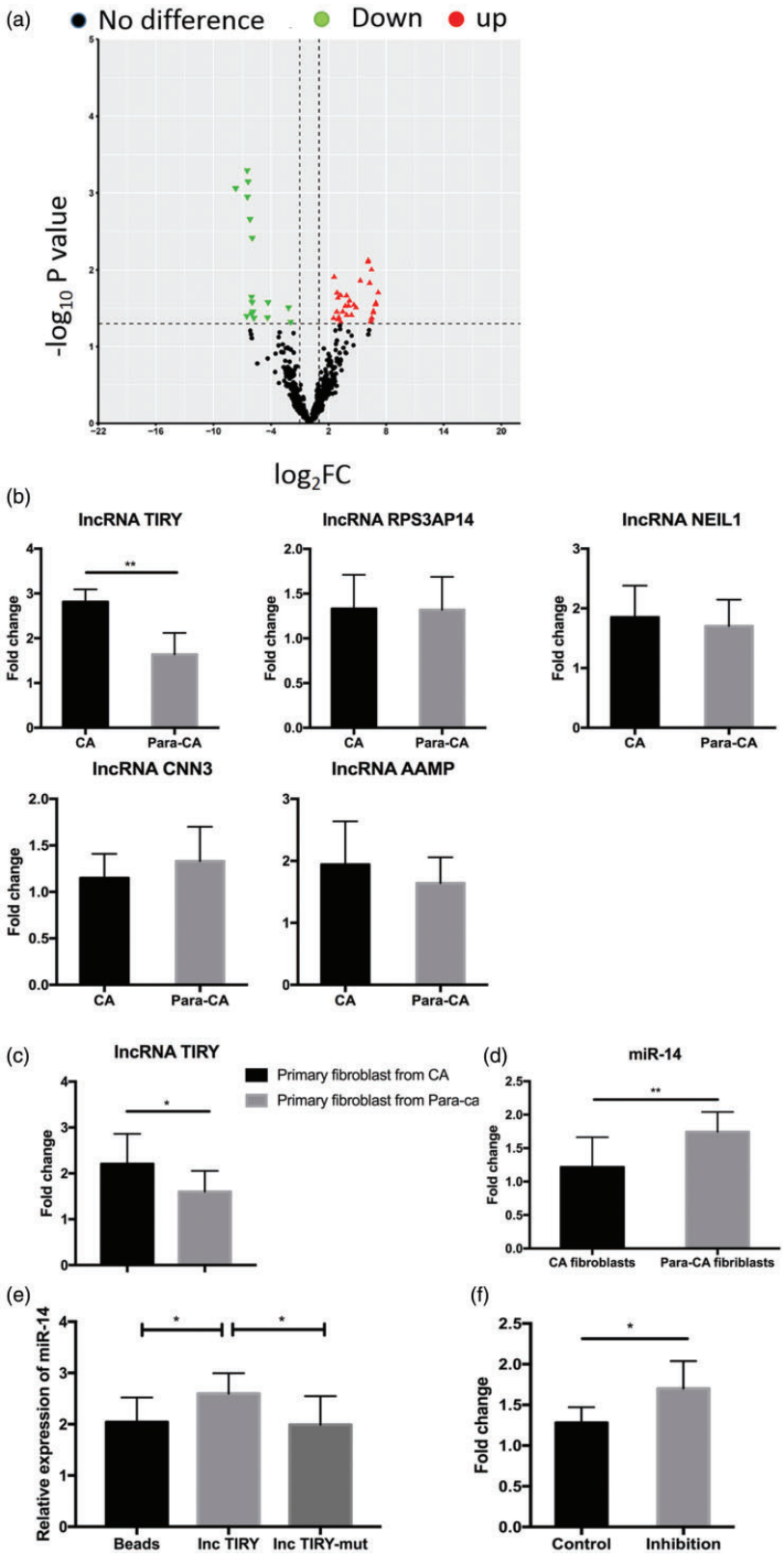


Figure 1. Increased TIRY expression and miR-14 downregulation in OSCC tissues. (a) Volcano plots illustrated that among differentially expressed circRNAs, 29 were upregulated and 17 were downregulated in GC tissues relative to normal tissues. (b) The comparison of potential lncRNAs with a significant difference between the OSCC and para-carcinoma tissues. Only TIRY expression was higher in OSCC tissues than that in para-carcinoma tissues. (c) The comparison of TIRY expression in cancer-associated fibroblasts and para-carcinoma tissue-derived fibroblasts. (d) miR-14 expression assessed using quantitative RT-PCR was significantly higher in the primary fibroblasts from OSCC tissues than in those from para-carcinoma tissues. (e) RNA pull-down assay showed that TIRY could bind to miRNA-14 in the primary CAFs. (f) qRT-PCR assays revealed that TIRY overexpression reduced miRNA-14 expression in the primary CAFs. All experiments were repeated at least five times. Data are represented as mean ± SD. Student's *t*-test was used for conducting statistical analyses. $P < 0.05$ indicated a significant difference. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

Table 1. The clinical baseline characteristics and demographic data of patients recruited in the prospective cohort.

	High-expression group ($n = 89$)	Low-expression group ($n = 65$)	*P* value
Gender			
Male	69 (77.5)	51 (78.5)	0.542
Female	20 (22.5)	14 (21.5)	
Age(years)			
≥60	55 (61.8)	49 (75.4)	0.143
<60	34 (38.2)	16 (24.6)	
Use of betel quid	68 (76.4)	54 (83.1)	
Clinical T stage			
T1/T2	43 (48.3)	21 (32.3)	0.091
T3/T4	36 (40.4)	44 (67.7)	
Clinical N stage			
N positive	30 (33.7)	15 (23.1)	0.106
N negative	59 (66.3)	54 (76.9)	
Pathological differentiation			
Well	61 (68.5)	40 (61.5)	0.271
Moderate/poorly	28 (31.5)	25 (38.5)	
Adjunctive radiotherapy	35 (39.3)	29 (44.6)	
OSF cases	76 (85.4)	57 (87.7)	0.421
Smoking			
Current/former	67 (75.3)	47 (72.3)	0.512
Never	22 (24.7)	18 (27.7)	

found that in CAF exosomes, miR-14 level decreased but Snail mRNA level was maintained (Figure 4(c) and (d)). Adding CAF exosomes to the TCA8113 medium unexpectedly increased Snail mRNA expression in TCA8113 cells (Figure 4(e)). Intriguingly, miR-14 expression was slightly but non-significantly increased after the delivery of additional TIRY-overexpressing CAF-derived exosomes (Figure 4(f)) within recipient OSCC cells. The media from CAFs treated with siRNA against TIRY led to a significant increase in miR-14 expression in recipient OSCC cells (Figure 4(f)). Our data illustrated that TIRY-overexpressing CAF-derived exosomes only delivered miR-14 to OSCC cells but elevated both Snail and miR-14 expressions in epithelial cancer cells, suggesting that the increased Snail expression may be due to the alteration of miR-14 expression in OSCC cells.

Administration of miR-14 mimics blocked CAF-derived exosome secretion and decreased the metastasis and invasion of OSCC

CAF-conditioned medium was centrifuged at 120,000 × *g* to remove exosomes and retain proteins. Using the transwell assay and wound healing test, we found that TCA8113 cells in CAF-conditioned medium treated with TIRY siRNA without exosomes had significantly higher invasion rate and motility than cells cultured in unconditioned medium (Figure 5(a) and (b)). Decreased miR-14 expression and increased Snail expression in TCA8113 cells suggested that blocking exosome secretion led to the promotion of EMT. These data show that physically removing CAF exosomes enhanced the invasion and metastasis of TCA8113 cells. We next sought to determine if the delivery of a miR-14 mimic would elicit the opposite effect. Such mimics were used to increase miR-14 levels in TCA8113 cells *in vitro* to determine their impact on metastasis and invasion. We found that the mimics significantly reduced Snail expression (Figure 5(c)) and decreased the metastasis and invasion of TCA8113 cells (Figure 5(d) and (e)). The miR-14 target mRNA was predicted using miRbase, and WNT3A was found to be the most likely candidate because it is the only gene involved in EMT through the Wnt/β-catenin signaling pathway in cancers. We then examined Wnt3A and β-catenin/p-β-catenin expressions in TCA8113 cells treated with media from CAF with or without TIRY overexpression. We found that the WNT/β-catenin signaling pathway was highly activated in TCA8113 cells treated with TIRY-overexpressing CAF-conditioned media compared with that in control cells. Significantly, increased Wnt3A expression and decreased β-catenin phosphorylation were observed in TCA8113 cells treated with TIRY-overexpressing CAF-conditioned medium (Figure 6(a) and (b)). DLRAs conducted in the TCA8113 cell line showed the specific binding of Wnt3A and miR-14 and the degradation of Wnt3A (Figure 6(c)). This finding revealed a loss of negative feedback of EMT, possibly due to the decreased delivery of miR-14 and consequently increased Wnt3A expression in OSCC cells.

Discussion

The tumor microenvironment, consisting of parenchymal cells and stromal cells, has recently been shown to reinforce the invasion and metastasis of cancer via an autocrine–paracrine communication. As one of the most abundant type of cells in tumor stroma, CAFs are some of the most predominant cell types present in the tumor microenvironment and can induce aggressive tumoral phenotypes in epithelial cancer cells.[15,16] In the present study, we demonstrated that CAFs isolated from OSCC tissues aggravate the invasion and metastasis of tumor because of the decreased delivery of miR-14 to epithelial cancer cells. TIRY knockdown may significantly reduce the invasion and metastasis of OSCC through the enhanced delivery of miR-14 to epithelial cancer cells.

CAFs present in cancer tissues usually play a vital role in supporting tumor growth and regulating biological processes via the secretion of cytokines and exosomes containing non-coding RNAs in an autocrine or paracrine manner[17]. For example, CAFs can secrete urokinase-type plasminogen activator to convert pro-MMPs to activated MMPs, resulting in increased angiogenesis and metastasis, as previously reported.[15] Besides their role in extracellular matrix remodeling, CAFs produce other cytokines such as FSP1 and hepatocyte growth factor to promote the metastasis of tumors, unlike para-carcinoma tissue-derived fibroblasts.[18] We found that CAFs express and secrete higher lncRNA levels than normal tissue-derived fibroblasts. lncRNAs are an important type of non-coding RNAs and can bind to miRNA, transcription factors, or proteins and regulate biological functions. Studies on lncRNAs provide novel insights for further understanding tumor biology; lncRNAs can be loaded and transported by exosomes to neighboring and distant cells, subsequently leading to a change in transcription or transcriptome profiles.

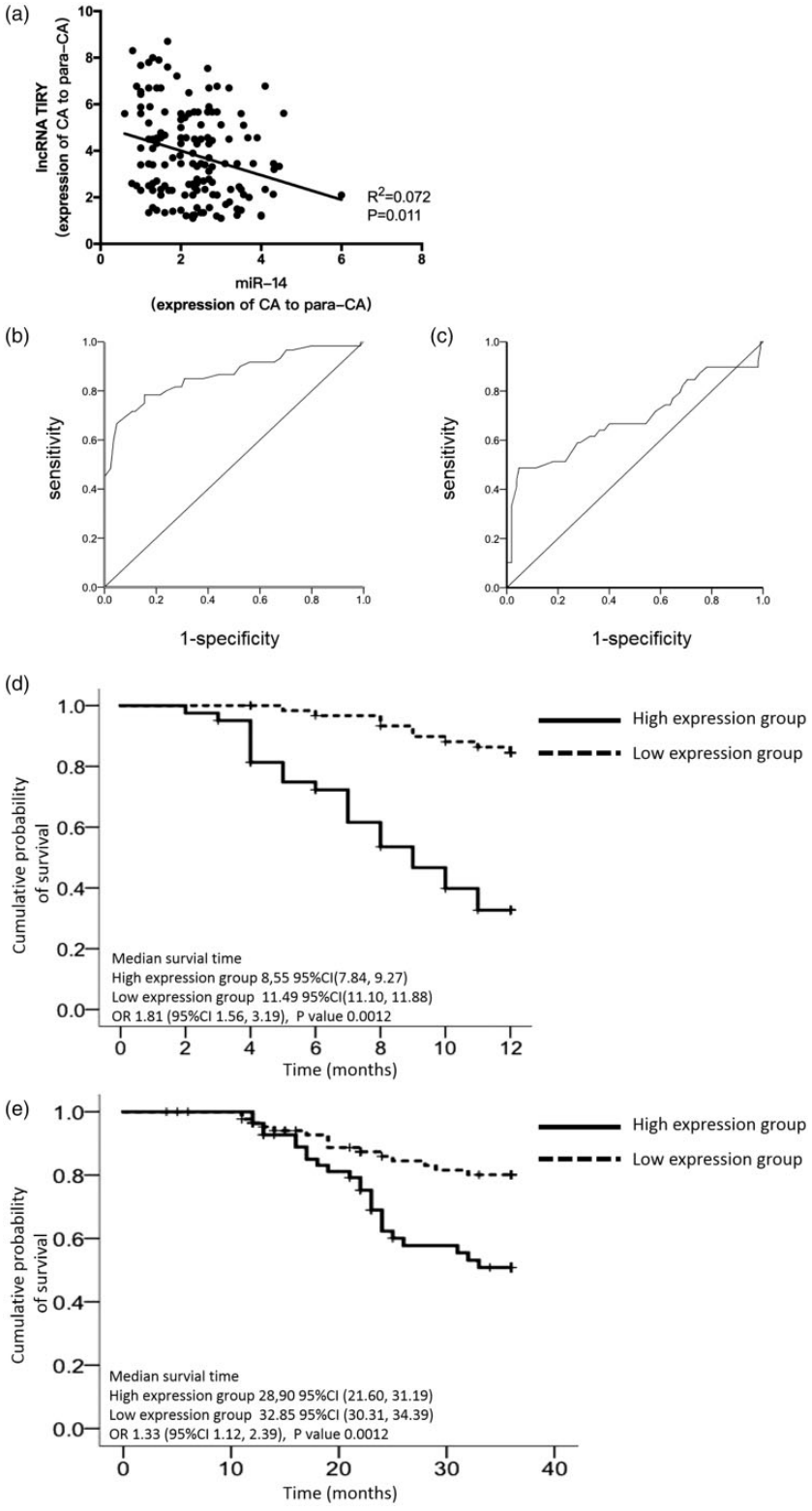
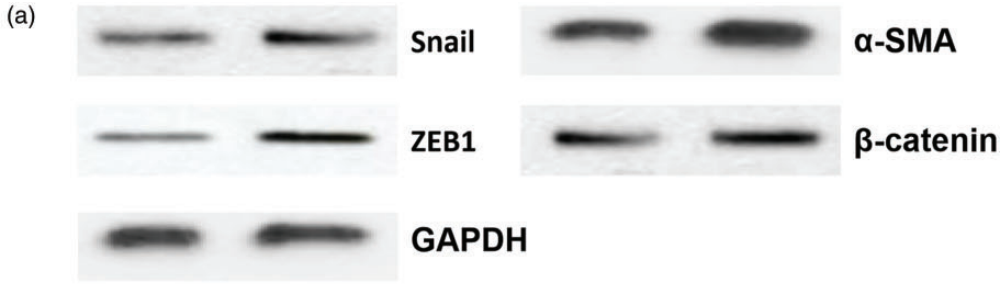
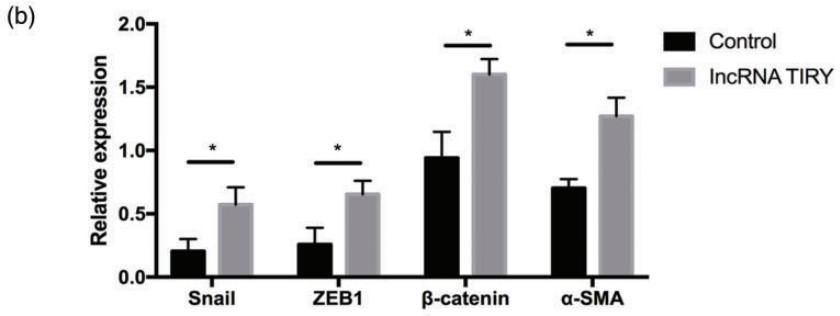
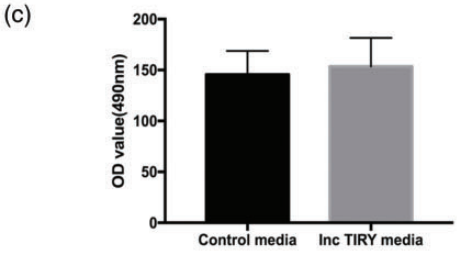
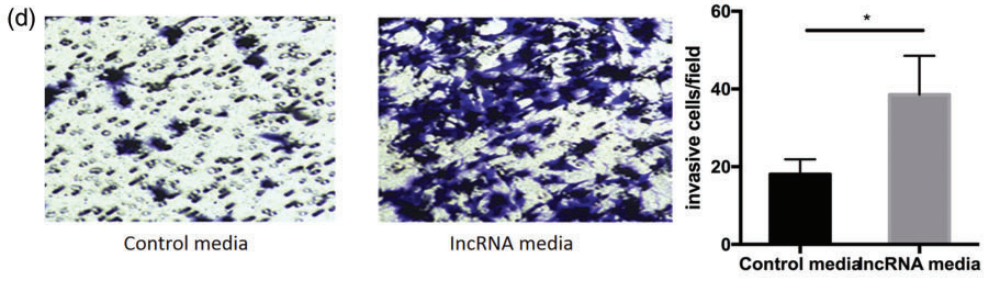


Figure 2. Higher TIRY expression in oral squamous cell carcinoma predicted poor prognosis. (a) Negative correlation of the ratio of TIRY and miR-14 expressions between carcinoma and para-carcinoma tissues was determined using linear regression analysis ($R^2 = 0.072$, $P = 0.011$). (b) Receiver operating characteristics curves demonstrated that the relative fluorescence intensity of TIRY measured using FISH assays could distinguish patients with a higher risk of recurrence or metastasis within one year of diagnosis from those with a lower risk (AUC = 0.897). (c) Receiver operating characteristics curves illustrated that the relative fluorescence intensity of TIRY measured using FISH assays could not distinguish patients with a higher risk of three-year mortality from those with a lower risk, as observed in one-year progression-free survival. (d and e) Grouping based on relative TIRY expression into high-expression and low-expression groups. Survival analysis demonstrated that patients with OSCC in the high-expression group had shorter progression-free survival (d) and overall survival (e) than those in the low-expression group. Kaplan–Meier analysis and log-rank tests were used for generating survival curves and comparison of survival between the two groups. Median survival and 95% confidence interval are shown at the bottom left. Survival analysis was performed using SPSS 24.0. $P < 0.05$ indicated a significant difference.









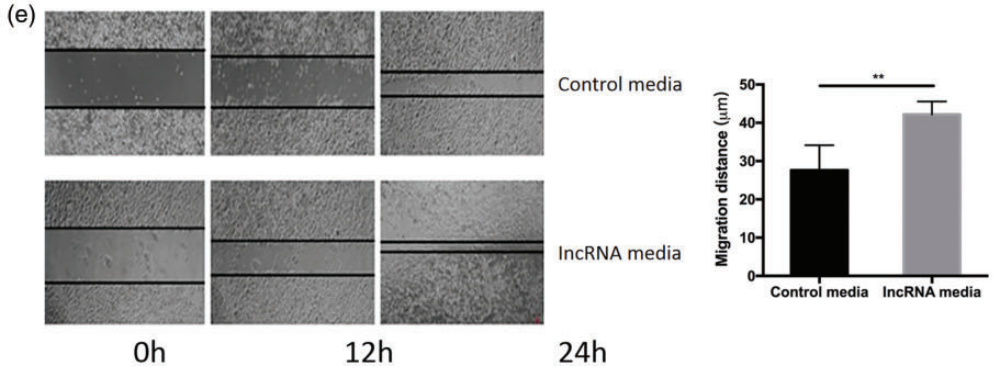


Figure 3. TIRY promoted EMT-related marker protein expression in cancer-associated fibroblasts and aggravated the invasion and metastasis of TCA8113 cells. (a) Representative data showing the results of immunoblot assays measuring expression of EMT marker proteins including EMT-related transcription factors (Snail and ZEB1) and cellular scaffolding proteins (α-SMA and β-catenin) in cancer-associated fibroblasts (CAFs). (b) The quantitative analysis of marker protein expressions. (c) MTT assays demonstrated that CAF-conditioned medium from primary TIRY-overexpressing CAFs has no effect on the proliferation of TCA8113 cells. (d and e) The results of the transwell assay and wound healing test demonstrated that CAF-conditioned media derived from TIRY-overexpressing primary CAFs enhanced the invasion and metastasis of TCA8113 cells. All experiments were repeated at least five times. Data are presented as mean $\pm$ SD. Student's t-test was used for conducting all statistical analyses. $P < 0.05$ indicated a significant difference. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (A color version of this figure is available in the online journal.)

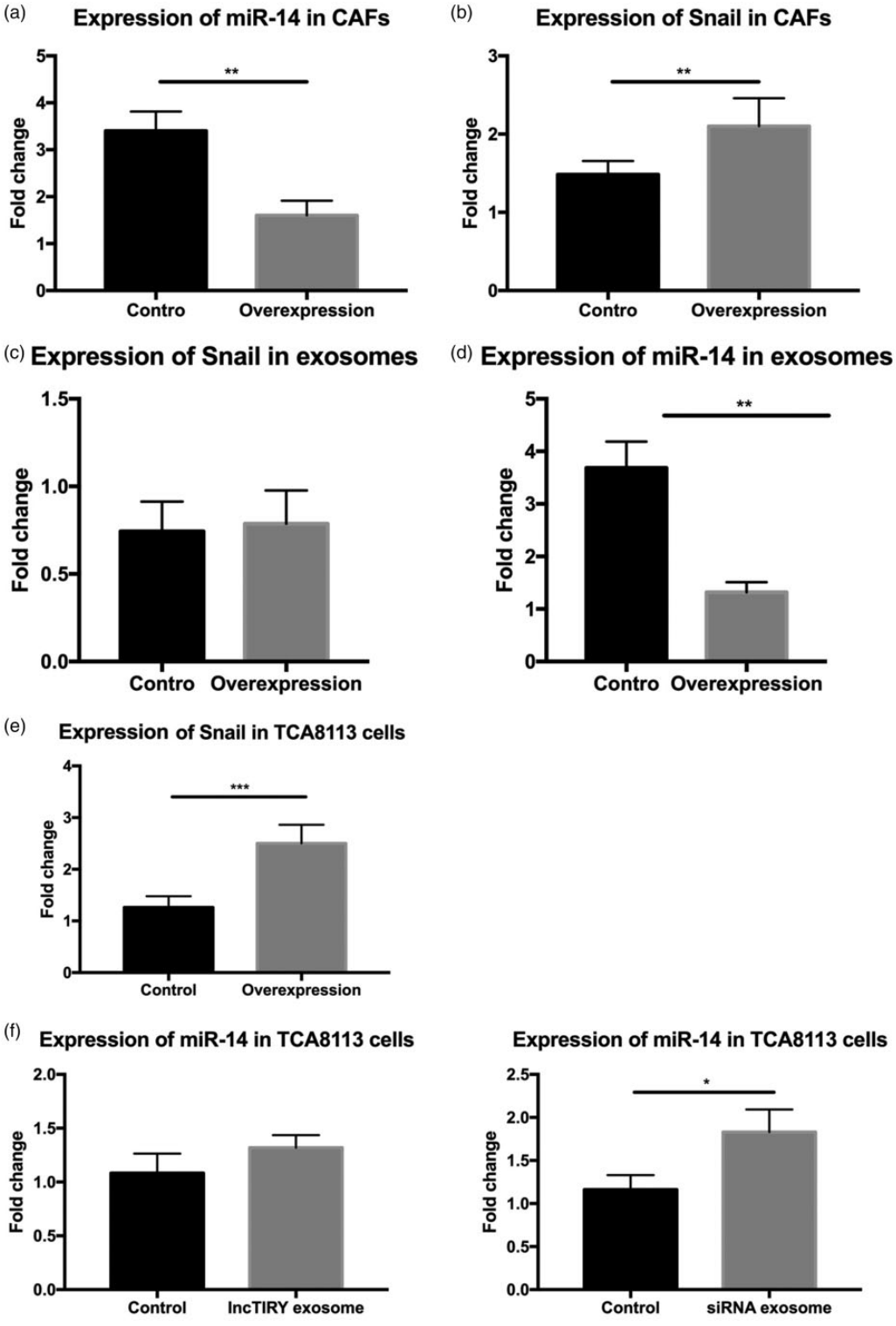


Figure 4. TIRY overexpression decreased the secretion of miR-14 and Snail in CAF-derived exosomes. TIRY overexpression led to lower miR-14 (a) and higher Snail expressions (b) in CAFs, as assessed using quantitative RT-PCR. (c and d) Compared with controls, TIRY-overexpressing CAF-derived exosomes were found to have decreased **miR-14** levels (d) and similar Snail mRNA levels (c). (e) The supply of CAF exosomes to TCA8113 cell media increased Snail mRNA expression in TCA8113 cells. (f) miR-14 expression is slightly non-significantly increased after the additional delivery of TIRY-expressing CAF-derived exosomes and is significantly increased in the recipient OSCC cells treated with TIRY siRNA-expressing CAF-conditioned media. All experiments were repeated at least five times. Data are presented as mean ± SD. Student's *t*-test was used for all statistical analyses. $P < 0.05$ indicated a significant difference. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

In our study, exosome-derived miR-14 had a negative effect on invasion and metastasis in the TCA8113 cell line in a paracrine manner and significantly downregulated Wnt3A expression in OSCC cells, suggesting that the loss of miR-14 due to TIRY overexpression in CAFs leads to the loss of feedback mechanisms mediated by the delivery of miR-14 to OSCC cells. We further examined whether the inhibition of TIRY in CAFs can cause miR-14 expression

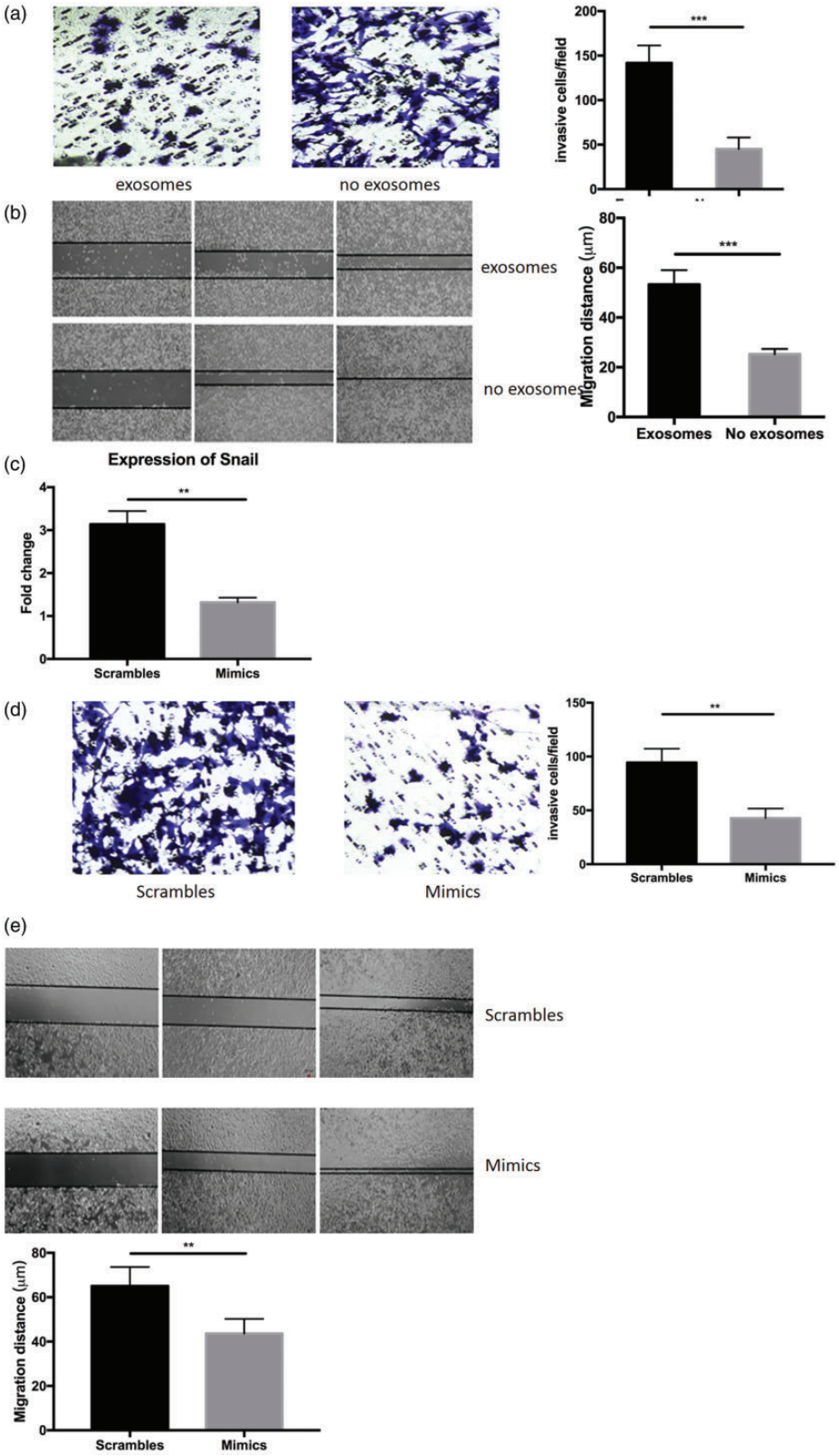


Figure 5. Administration of miR-14 mimics blocked CAF-derived exosome secretion and decreased metastasis and invasion. (a and b) The removal of exosomes from TIRY siRNA-expressing CAFs-conditioned media led to a significant increase in invasiveness and metastasis of TCA8113 cells in the transwell assay (a) and wound healing test (b). (c) miR-14 mimics increased miR-14 expression in TCA8113 cells *in vitro*. (d and e) The administration of miR-14 mimics significantly decreased metastasis and invasion as assessed using the transwell assay and wound healing test. All experiments were repeated at least five times. Data are presented as mean ± SD. Student's *t*-test was used for all statistical analyses. $P < 0.05$ indicated a significant difference. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

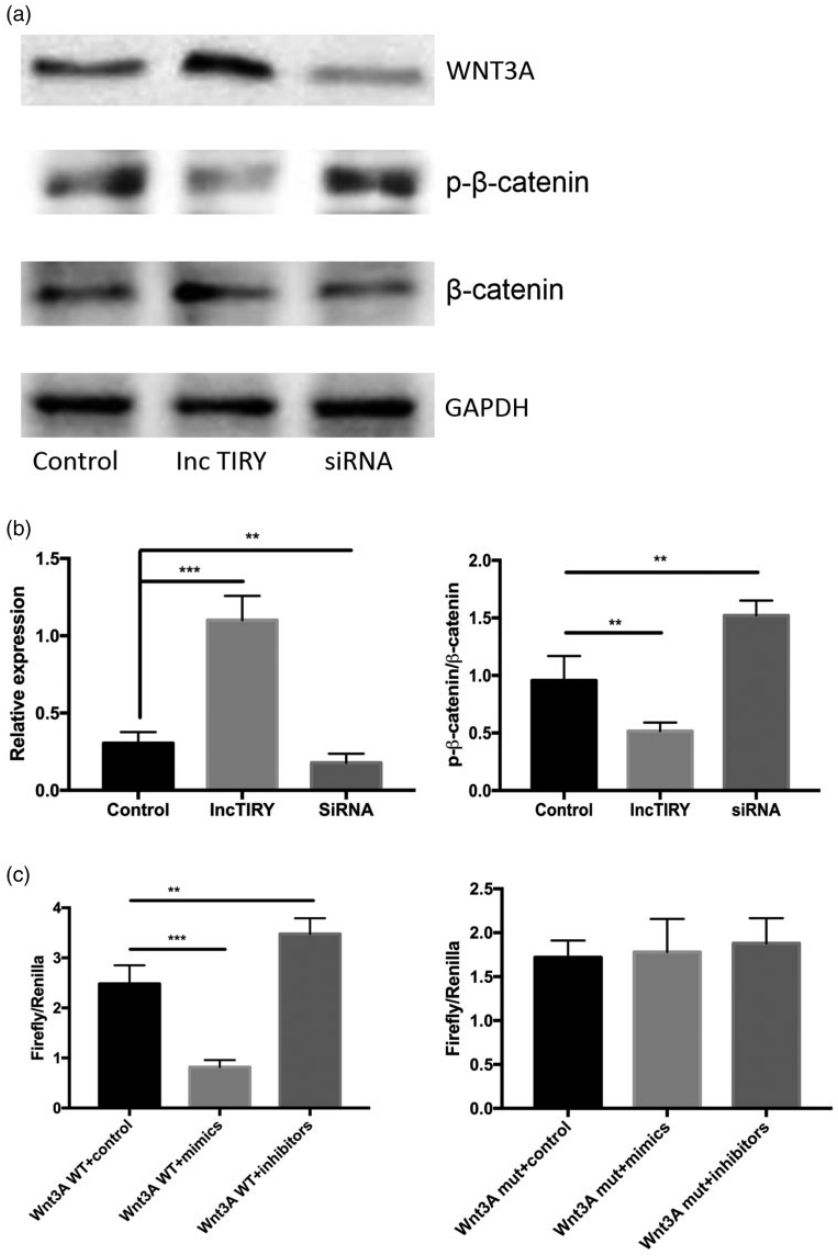


Figure 6. Activation of the Wnt/β-catenin signaling pathway in TCA8113 cells treated TIRY-overexpressing CAF-conditioned media. (a) Representative immunoblot showing the expression of proteins in the Wnt/β-catenin signaling pathway. (b and c) The quantitative analysis of the relative expression of proteins in the Wnt/β-catenin signaling pathway. (d) Dual-luciferase reporter assays demonstrated the binding of miR-14 and Wnt 3A in the TCA 8113 cell line. All experiments were repeated at least five times. Data are presented as mean $\pm$ SD. Student's *t*-test was used for all statistical analyses. $P < 0.05$ indicated a significant difference. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

changes in OSCC cells and decrease cancer progression. We found that the administration of siRNA against TIRY in CAFs may improve the aggressive biological phenotype of TCA8113 cells. Moreover, we showed that TIRY expression was negatively correlated with miR-14 transcription levels and was associated with poor prognosis in OSCC specimens. Functionally, TIRY was not only essential for increased EMT in CAFs but also critical for enhancing the invasion and metastasis of tumor parenchymal cells. The deficiency of miR-14 in tumor parenchymal cells due to decreased miR-14 level in exosomes may lead to the activation of the Wnt/β-catenin signaling pathway and may increase EMT in epithelial cancer cells, further enhancing invasion, metastasis, and other biological phenotypes. These data suggest that TIRY functions as a key molecule in the promotion of EMT in CAFs and of an aggressive phenotype of tumor parenchymal cells.

A previous study analyzed the association between CAFs and angiogenesis and matrix remolding in OSCC and found that α-SMA expression, tumor invasion, recurrence risk, and lymph node involvement were closely related to each other.[8,19,20] A recent study suggested that CAFs may promote the invasion and metastasis of OSCC by increasing MMP-9 expression and may promote angiogenesis *in vivo*.[8] Although previous studies have demonstrated an association between CAFs and tumor aggressiveness in

OSCC, the underlying molecular mechanisms and crosstalk between CAFs and tumor parenchymal cells are still unclear. In the present study, we found that the dysregulation of miR-14 expression in CAF-derived exosomes was involved in the aggressiveness of tumor parenchymal cells. This was possibly due to aberrant TIRY overexpression leading to the activation of the Wnt/β-catenin signaling pathway, thus promoting EMT and tumor aggressiveness.

In conclusion, our results suggest that TIRY leads to increased EMT in CAFs and blocks the transport of miR-14 from CAFs to epithelial cancer cells, which manifests as a decrease in miR-14 level in CAFs-derived exosomes. The consequent decreased delivery of miR-14 enhances the progression and metastasis of OSCC. Thus, TIRY in CAFs may be a valuable predictive biomarker and promising therapeutic target in OSCC.

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SUPPLEMENTAL MATERIAL

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