miR-629 Targets TRIM33 to Promote TGFβ/Smad Signaling and Metastatic Phenotypes in ccRCC

Kentaro Jingushi1, Yuko Ueda1, Kaori Kitae1, Hiroaki Hase1, Hiroshi Egawa1, Ikumi Ohshio1, Ryoji Kawakami1, Yuri Kashiwagi1, Yohei Tsukada1, Takumi Kobayashi1, Wataru Nakata2, Kazutoshi Fujita2, Motohide Uemura2, Norio Nonomura2, and Kazutake Tsujikawa1

Abstract

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney, and clear cell RCC (ccRCC) represents its most common histological subtype. To identify a therapeutic target for ccRCC, miRNA expression signatures from ccRCC clinical specimens were analyzed. miRNA microarray and real-time PCR analyses revealed that miR-629 expression was significantly upregulated in human ccRCC compared with adjacent noncancerous renal tissue. Functional inhibition of miR-629 by a hairpin miRNA inhibitor suppressed ccRCC cell motility and invasion. Mechanistically, miR-629 directly targeted tripartite motif-containing 33 (TRIM33), which inhibits the TGFβ/Smad signaling pathway. In clinical ccRCC specimens, downregulation of TRIM33 was observed with the association of both pathologic stages and grades. The miR-629 inhibitor significantly suppressed TGFβ-induced Smad activation by upregulating TRIM33 expression and subsequently inhibited the association of Smad2/3 and Smad4. Moreover, a miR-629 mimic enhanced the effect of TGFβ on the expression of epithelial–mesenchymal transition-related factors as well as on the motility and invasion in ccRCC cells. These findings identify miR-629 as a potent regulator of the TGFβ/Smad signaling pathway via TRIM33 in ccRCC.

Implications: This study suggests that miR-629 has biomarker potential through its ability to regulate TGFβ/Smad signaling and accelerate ccRCC cell motility and invasion. Mol Cancer Res; 13(3): 565–74. © 2014 AACR.

Introduction

Renal cell carcinoma (RCC) represents the leading cause of cancer death among urological malignancies (1), and clear cell RCC (ccRCC) is its most common histological subtype. Early-stage ccRCC is curable by surgery; however, locally advanced or metastatic cancer is chemotherapy resistant. Recently, molecular-targeted drugs, including the tyrosine kinase inhibitors sunitinib and sorafenib, and the mTOR inhibitors temsirolimus and everolimus are used for the treatment of ccRCC; however, their effect is restrictive (2). Therefore, it is necessary to search a novel molecular target to improve the therapeutic cues of advanced ccRCC.

TGFβ/Smad pathway is known to be a key player in cell proliferation, differentiation, apoptosis, and epithelial–mesenchymal transition (EMT) in various cancer cells (3, 4). In ccRCC cells, inhibition of TGFβ/Smad pathway attenuates cell migration and invasion but has no direct effect on cell proliferation (5). The expression of TGFβ is correlated with pathologic stages and grades, and it promotes the establishment of bone metastasis in RCC (6, 7). Although some reports have shown the potential role of the TGFβ/Smad pathway on ccRCC progression, its precise regulation mechanism in ccRCC progression remains largely unknown.

Tripartite motif-containing 33 (TRIM33) is a member of the tripartite motif superfamily that possess a Really Interesting New Gene (RING) domain, 2 B-boxes, and a coiled-coil domain at the N-terminus as well as a plant homeodomain (PHD) and a bromo domain at the C-terminus (8). TRIM33 is implicated in the transcriptional regulation during hematopoiesis (9, 10). TRIM33 is implicated in the transcriptional regulation during hematopoiesis (9, 10). TRIM33 was implicated in the transcriptional regulation during hematopoiesis (9, 10). TRIM33 was implicated in the transcriptional regulation during hematopoiesis (9, 10). TRIM33 is implicated in the transcriptional regulation during hematopoiesis (9, 10). TRIM33 is implicated in the transcriptional regulation during hematopoiesis (9, 10).

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: Kentaro Jingushi, Osaka University, 1-6 Yamanadaoka, Suita, Osaka, 565-0871, Japan. Phone: 81-6-6879-8192; Fax: 81-6-6879-8190; E-mail: jingushi-kk@ips.osaka-u.ac.jp

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Materials and Methods

Chemicals and antibodies
Polyclonal anti-TRIM33 antibody, monoclonal anti-Smad2/3 antibody, monoclonal anti-phospho-Smad2/3 antibody (Ser465/467, Ser423/425), polyclonal anti-N-cadherin antibody, monoclonal anti-E-cadherin antibody, monoclonal anti-Snail antibody, monoclonal anti-Slug antibody, monoclonal anti-ZEB1 antibody, and monoclonal anti-HNF4α antibody were purchased from Cell Signaling Technology. Monoclonal anti-β-tubulin antibody was purchased from Sigma. Monoclonal anti-Smad4 antibody was purchased from Abcam. Monoclonal lamin A/C antibody was purchased from BD Transduction Laboratories. Recombinant human TGFβ1 was purchased from Wako Pure chemical (Japan). TGFβ1 was used at a concentration of 10 ng/mL in the experiments.

Clinical specimens
ccRCC specimens were obtained from patients while they underwent primary curative resection at the Osaka University Medical Hospital, Japan. Tumor-associated normal renal tissue was also obtained from a subset of these patients when possible. Histological diagnosis was established for standard hematoxylin and eosin–stained sections by two senior pathologists experienced in RCC diagnosis. Tumors were staged according to the 6th AJCC TNM staging system and graded according to Fuhrman’s nuclear grading system. Prior written and informed consent was obtained from each patient, and the study was approved by the ethics review board of the Osaka University Medical Hospital.

Analysis by miRNA microarray and quantitative real-time PCR
Following excision, the tissue specimens were immediately immersed in RNAlater (QIAGEN) and stored at −20°C until RNA extraction. miRNAs were purified using the mirNeasy mini Kit (QIAGEN). miRNA analysis was conducted using 9 matched-pair samples plus 7 tumor specimens on miRNA microarray 2.0 (Affymetrix). The arrays were scanned using the Affymetrix GeneChip Scanner 3000, and the scanned data were processed using the Agilent GeneSpring GX software (GEO accession number: GSE55138).

A real-time PCR analysis was conducted to validate the miR-629 expression in 32 matched-pair samples of ccRCC by using the Mir-X miRNA First-Strand Synthesis Kit (Clontech) in duplicate. Thermal cycling conditions included an initial step at 98°C for 30 seconds and 40 cycles at 95°C for 2 seconds and at 63°C for 5 seconds by using a miR-629–specific primer (5'-tggttaactgtgagacact-3') and a U6 snRNA–specific primer (Clontech) as an internal control. A correlation analysis between miR-629 and TRIM33 mRNA expression (fold change: tumor vs. normal) was conducted using 41 matched-pair samples. For the correlation analysis, the total RNA was isolated by using Trizol reagent (Invitrogen). The PrimeScript RT reagent Kit (Takara) was used to prepare cDNA from 500 ng total RNA. mRNA and miRNA expression was quantified as delta Ct values.

Clinical and pathologic data related to the clinical samples are presented in Table 1.

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>For miRNA-microarray analysis</th>
<th>For real-time PCR analysis (validation of miR-629 expression)</th>
<th>For real-time PCR analysis (correlation analysis)</th>
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<td>Range</td>
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Western blotting
Cells were lysed with Laemmli SDS sample buffer containing 5% 2-mercaptoethanol. Protein samples were separated on a 7.5% to 15% SDS–PAGE gel and then transferred to a polyvinylidene difluoride membrane by using the Bio-Rad semidry transfer system (1 hour, 12 V). Immunoreactive proteins made to react with the antibodies described previously were visualized by treatment with a detection reagent (ECL, Prime Western blotting detection reagent, GE Healthcare). Densitometric analysis was performed using the NIH Image J software.

Luciferase reporter assay
A pmirGLO dual-luciferase miRNA target expression vector was used for the 3'-untranslated region (3′UTR) luciferase reporter assay (Promega). The following sequences of oligos were used for the construction of the pmirGLO/TRIM33 3′-UTR vector: 5′-ctgggagctggttaaatctct-3′ and 5′-tggttaacagtatgcctagagct-3′. For the TGFβ1/Smad reporter assay, the cells were transfected with pGL4.48 vector containing 3 tandem copies of a Smad-binding element (SBE), which drives the transcription of the firefly luciferase gene (Promega), by using Lipofectamine 2000 (Life Technologies). The cells were cultured for 24 hours following by stimulation with TGFβ1 for 24 hours.

Table 1. Clinical and pathological data

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Luciferase activity was determined using a luminometer (Turner Biosystems 20/20 n luminometer; Promega).

Wound-healing assay
Caki-2 cells or ACHN cells transfected with the miRIDIAN hairpin inhibitor or mimic or siRNA were seeded in a 24-well plate (Caki-2 cells: 2.0 × 10⁴ cells/well; ACHN cells: 4.0 × 10⁴ cells/well) and incubated for 72 hours. A wound was created in a monolayer of about 90% confluent cells by using a sterile 1-ml pipette tip. Cell pictures were recorded at 0 and 12 hours for Caki-2 cells and at 0 and 24 hours for ACHN cells after wound creation by using an Olympus IX71 fluorescence microscope.

Water-soluble tetrazolium salt-1 cell growth assay
Caki-2 cells or ACHN cells transfected with the miRIDIAN hairpin inhibitor or mimic were reseeded in a 96-well plate (Caki-2 cells: 0.5 × 10⁴ cells/well; ACHN cells: 0.3 × 10⁴ cells/well) and incubated for indicated time. After incubation for 2 hours with water-soluble tetrazolium salt-1 (WST-1) reagent (Dojindo) at 37°C and 5% CO₂, the optical density was read at a wavelength of 450/630 nm (Ex/Em).

Cell invasion assay
The BioCoat tumor invasion system (BD Bioscience) with the 8.0-μm pore size FluoroBlok membrane was used to perform the cell invasion assay. Caki-2 cells or ACHN cells transfected with the miRIDIAN hairpin inhibitor or mimic or siRNA for 72 hours were reseeded in the insert of 96-well plate (2 × 10⁴ cells/well) in serum-free conditions, and medium supplemented with 10% FBS was used as a chemoattractant in the base plate. Following incubation for 12 hours, the cells were labeled with calcein AM (4 μg/mL), and the fluorescence of the invaded cells was read at a wavelength of 494/517 nm (Ex/Em).

miRNA hairpin inhibitor, mimic, and siRNA transfection
miRIDIAN miRNA hairpin inhibitor or mimic negative control and miRIDIAN miRNA hairpin inhibitor or mimic for human hsa-miR-629 (MMAT10004810/MM1003643) were purchased from Thermo Scientific Dharmacon. The miRIDIAN miRNA hairpin inhibitors and mimics were transfected at a concentration of 50 nmol/L in the experiments using Lipofectamine RNAiMAX reagent. siRNA duplexes used to downregulate TRIM33 mRNA (TRIM33 MISSION siRNA #1: CUGAUCAGGUGAAGGUCAATT, TRIM33 MISSION siRNA #2: GACAGAUGAAGUUACUACATT) were purchased from Sigma Aldrich. Negative control siRNA duplex (siRNA sequence: AGTAAAGAAGCCTGCGTGGTCTT) was purchased from B-Brige. For all siRNA transfection studies, 50 nmol/L siRNAs were transfected using Lipofectamine RNAiMAX reagent.

Immunohistochemistry
The expression of TRIM33 and hepatocyte nuclear factor 4α (HNF4α) was determined by immunohistochemical staining of the paraffin-embedded tissue of normal kidney (one sample) and ccRCC (19 samples for TRIM33 and 17 samples for HNF4α). Formalin-fixed paraffin-embedded sections (5 μm in thickness) were deparaffinized and rehydrated. After the slides were steamed for 20 minutes in 10 mmol/L citrate buffer (pH 6.0) for antigen retrieval, endogenous peroxidase was blocked using 3% H₂O₂. Immunohistochemical staining for TRIM33 and HNF4α was performed using anti-TRIM33 (1:500) and anti-HNF4α antibodies (1:500), respectively, and the EnVision®+ Detection System (DAKO) was used, according to the manufacturer’s instructions. Primary antibodies were incubated overnight at 4°C and counterstained with hematoxylin. The levels of TRIM33 and HNF4α staining were classified into 3 groups according to the score of the positive cells: 2, strong; 1, moderate; and 0, weak. TRIM33 and HNF4α staining indices were determined as the sum of the immunostained cells per 200 cells × 3 fields per section.

Statistical analysis
The results were expressed as the mean ± SD of the mean or median. Differences between the values were statistically analyzed using the Student t test or one-way ANOVA with Bonferroni post-hoc tests and correlation between the values were statistically analyzed using the Pearson correlation analysis (GraphPad Prism 6.0; GraphPad software). A P value of <0.05 was considered statistically significant.

Results
MiRNA expression signature in ccRCC
To obtain the miRNA expression signature in ccRCC, we performed miRNA microarray analysis using ccRCC specimens. We identified unique miRNA expression signatures for ccRCC. High expression of miRNAs, including that of miR-122, miR-21, miR-210, miR-155, and miR-629, was confirmed in ccRCC (Supplementary Table S1). From the viewpoint of novelty, we focused on miR-629. To determine the expression of miR-629 in the ccRCC specimens, we performed quantitative real-time PCR analysis. As shown in Fig. 1A and B, the expression of miR-629 was 4.4-fold higher in the ccRCC tissues than in the adjacent normal renal tissues; however, there was no significant difference in the expression among the different pathologic stages and grades.

MiR-629 regulates cell migration and invasion in Caki-2 cells
To investigate the role of the upregulated miR-629 in ccRCC, we first examined the expression level of miR-629 in the ccRCC cell lines. Among the 4 ccRCC cell lines examined, the expression of miR-629 was the highest in the Caki-2 cells (Fig. 2A). Therefore, we analyzed the function of miR-629 by using the Caki-2 cells. To investigate the potential function of miR-629, we examined the effect of the miR-629 inhibitor and mimic on cell growth, motility, and invasion in Caki-2 and ACHN cells. Although it does not have a significant effect on cell growth (Fig. 2B), the miR-629 inhibitor significantly reduced the motility (Fig. 2D) and invasion ability (Fig. 2F) of the Caki-2 cells. Conversely, the miR-629 mimic increased the cell growth (Fig. 2C) and invasion ability (Fig. 2G) of the ACHN cells. Interestingly, transfection of miR-629 mimic promoted the scattered cell phenotype compared with negative control mimic-transfected ACHN cells (Fig. 2E). These results suggest that highly expressed miR-629 upregulates the cell proliferation, motility, and invasion abilities of the ccRCC cells.

MiR-629 targets TRIM33 in ccRCC cells
To identify a target of miR-629 in ccRCC cells, we utilized target prediction programs (miRbase, TargetScan, and miRanda) and found that TRIM33, which is an inhibitor of the TGFβ signaling pathway (10–12), was a potential target. The target prediction programs predicted that the 3′-UTR of TRIM33 mRNA contained complementary sequence for the seed region of miR-629 (Fig. 3A). To confirm whether TRIM33 was a target of miR-629, we cotransfected both miR-629 inhibitor and luciferase reporter
vector with a sequence containing the predicted miR-629–binding site within the human TRIM33 3′-UTR in the Caki-2 cells. As shown in Fig. 3B, decreased luciferase activity was detected in the transfection of the vector with TRIM33 3′-UTR compared with that of the control vector. The cotransfection of the vector with TRIM33 3′-UTR and the miR-629 inhibitor significantly increased the luciferase activity in comparison with that observed in the negative control miRNA inhibitor. Conversely, compared with the negative control miR-629 mimic, the miR-629 mimic significantly decreased the luciferase activity (Fig. 3C). Moreover, the miR-629 inhibitor upregulated the expression of TRIM33 at the protein level in the Caki-2 cells (Fig. 3D), and miR-629 mimic significantly reduced TRIM33 expression at both the protein (Fig. 3E) and mRNA levels (Supplementary Fig. S1A). These results indicated that TRIM33 is a target of miR-629 in the ccRCC cells. To examine the expression of TRIM33 in ccRCC, formalin-fixed paraffin-embedded clinical specimens from patients with ccRCC were immunohistochemically stained with anti-TRIM33 antibody. TRIM33 was strongly expressed in the nuclei of normal kidney tissues, whereas almost no expression was observed in the ccRCC tissues (Fig. 3F). Moreover, the low expression of TRIM33 was observed for the higher pathologic stages and grades (Fig. 3F). Interestingly, a weak inverse correlation \( r = -0.23 \) between miR-629 and TRIM33 mRNA levels in ccRCC specimens was observed (Supplementary Fig. S1B).

Recently, miR-629 was identified as one of the regulators that link the inflammatory response to hepatocyte transformation in human hepatocellular carcinoma by downregulating the transcription factor HNF4α \( (17) \). In ccRCC, the expression and DNA-binding activity of HNF4α are reported to be frequently reduced compared with that in the normal kidney tissue \( (18, 19) \). To determine whether HNF4α was downregulated by miR-629 in ccRCC cells, we verified the effect of the miR-629 inhibitor on HNF4α expression. The miR-629 inhibitor showed no significant effect on HNF4α expression in Caki-2 cells (Supplementary Fig. S1A). Moreover, there was no correlation between miR-629 (Fig. 2A) and HNF4α (Supplementary Fig. S5B) expression in ccRCC cell lines. However, HNF4α expression was significantly downregulated in ccRCC specimens, and inversely correlated with the pathologic stages and grades of ccRCC (Supplementary Fig. S5C and S5D). Therefore, it seems that downregulated HNF4α expression in ccRCC occurs irrespective of miR-629 regulation.

miR-629 upregulates the intensity of TGFβ/Smad signaling pathway

Since TRIM33 is reported to inhibit the TGFβ signaling pathway, we focused on the function of miR-629 in the TGFβ signaling pathway. To investigate the effect of the miR-629 on Smad-induced transcription activity, we used a luciferase reporter vector containing SBE. As shown in Fig. 4A, the miR-629 mimic significantly increased the luciferase transcriptional activation triggered by TGFβ1 in ACHN cells. Conversely, the miR-629 inhibitor markedly suppressed the luciferase transcriptional activation triggered by TGFβ1 (Fig. 4B). The suppressive effect of the miR-629 inhibitor on the Smad activation by TGFβ1 was attenuated by TRIM33 siRNAs. The knockdown effects of TRIM33 siRNAs were confirmed by the Western blot analysis (Supplementary Fig. S2). These results suggest that TRIM33 would be a crucial regulation molecule in the TGFβ-induced Smad activation via miR-629. To verify the role of TRIM33 on the TGFβ signaling pathway in ccRCC cells, we investigated the relationship between the intensity of the TGFβ signal transduction and TRIM33 expression levels in the ccRCC cell lines. TRIM33 was found to be differentially expressed in the four ccRCC cell lines examined, with a inverse relationship with the miR-629 expression levels in ccRCC cell lines (Fig. 4C). Moreover, the fold response of Smad activation induced by TGFβ1 showed an inverse correlation \( r = -0.87 \) with the highest expression being observed in the Caki-2 cells among the other ccRCC cell lines (Fig. 4D). To further confirm the effect of TRIM33 on the TGFβ signaling, TRIM33 knockdown was induced in ACHN cells, which show a relatively high expression of TRIM33 (Supplementary Fig. S3A and S3B). Smad activity induced by TGFβ1 addition was further enhanced by the transfection of TRIM33 siRNAs. Collectively, these results indicate that miR-629 upregulates TGFβ1-induced Smad activation through the downregulation of TRIM33 expression, and the TGFβ signal intensity may be related to TRIM33 and miR-629 expression levels in the ccRCC cells.

MiR-629 upregulates the intensity of TGFβ/Smad signaling pathway by facilitating Smad2/3 and Smad4 association in ccRCC cells

TRIM33 has been shown to inhibit the activation of the TGFβ signaling pathway by competing with Smad4 for binding to Smad2/3 \( (10, 12) \) or by targeting Smad4 expression and cellular localization \( (11) \). Therefore, we investigated the effect of the miR-629 inhibitor on Smad2/3 or Smad4 functions in the Caki-2 cells.
miR-629 upregulates the migration and invasion in Caki-2 cells. A, expression of miR-629 in ccRCC cell lines was examined by quantitative real-time PCR in duplicate. Data are the relative expression normalized to U6 snRNA. B, Caki-2 cells transfected with the miR-629 inhibitor or negative control (NC) miRNA inhibitor for 24 hours were reseeded in a 96-well plate (0.5 × 10^4 cells/well) and incubated for the indicated times and examined by the WST-1 assay. Values are presented as mean ± SD of six independent experiments. C, ACHN cells transfected with the miR-629 mimic or the negative control miRNA mimic for 24 hours were reseeded in a 96-well plate (0.3 × 10^4 cells/well) and incubated for the indicated times and examined by the WST-1 assay. Values are presented as mean ± SD of six independent experiments. ***, P < 0.001 versus negative control mimic. D, Caki-2 cells were transfected with the miR-629 inhibitor or negative control miRNA inhibitor for 72 hours. Cell motility was measured 12 hours after a wound was formed by scraping. Representative results of cell motility in the scratch wound-healing assay are shown. The results are expressed as mean ± SD of four independent experiments. ***, P < 0.01 versus control inhibitor. White bars, 200 μm. E, ACHN cells were transfected with the miR-629 mimic or negative control miRNA mimic for 72 hours. Cell motility was measured 24 hours after wound formation by scraping in serum-free conditions. Representative results for cell motility in the scratch wound-healing assay are shown. The results are expressed as mean ± SD of six independent experiments. White bars, 200 μm. F, Caki-2 cells and ACHN cells were transfected with the miR-629 inhibitor or negative control miRNA inhibitor (F) or with the miR-629 mimic or negative control miRNA mimic (G) for 72 hours. The transfected cell suspension was added to the upper chamber of Matrigel-coated transwell membrane inserts, and the lower chamber was filled with the medium and then cultured for 12 hours. Fluorescence derived from invasive cells was measured. Values are mean ± SD of three independent experiments. **, P < 0.01 versus control inhibitor.

cells. We first evaluated the effect of the miR-629 inhibitor on the cellular localization of Smad2/3 and Smad4 by immunofluorescent staining as well as Western blot analyses with anti-Smad2/3 and anti-Smad4 antibodies. As shown in Fig. 5A–C, although neither TGFβ1 nor the miR-629 inhibitor alone had an obvious effect on Smad4 cellular localization or Smad4 protein amount, Smad2/3 translocation from the cytosol to the nucleus, triggered by TGFβ1, was diminished by the miR-629 inhibitor. TRIM33 knockdown attenuated the effect of the miR-629 inhibitor on Smad2/3 nuclear localization triggered by TGFβ1 (Fig. 5B). Moreover, the phosphorylation level of Smad2/3 was unaffected by the miR-629 inhibitor (Fig. 5C and D). To clarify the suppressive mechanism of Smad activity by the miR-629 inhibitor, Caki-2 cells stimulated with or without TGFβ1 were immunoprecipitated with anti-Smad2/3 and immunoblotted with anti-Smad2/3, anti-phospho-Smad2/3, and anti-Smad4 antibodies. As shown in Fig. 5E, TGFβ1 induced the association of Smad2/3 and Smad4. Interestingly, the association was diminished in the miR-629 inhibitor–transfected Caki-2 cells. Moreover, TRIM33 knockdown weakly attenuated the effect of the miR-629 inhibitor on the TGFβ1–induced association of Smad2/3 and Smad4. These results suggest that miR-629 promotes TGFβ signal transduction by downregulating TRIM33 expression and by subsequently facilitating the association of Smad2/3 and Smad4 in the ccRCC cells.
The effect of the miR-629 on the expression of EMT-related factors

Since the TGFβ signaling pathway is known as a major inducer of the EMT, features downregulation of E-cadherin expression and upregulation of N-cadherin expression leading to facilitation of cellular migration and invasion activities (20), we investigated whether miR-629 could regulate the expression of EMT-related molecules induced by TGFβ1 treatment. As shown in Fig. 6A, although TGFβ1 induced the expression of EMT-related molecules (e.g., N-cadherin, E-cadherin, Slug, Snail, and ZEB1), the miR-629 inhibitor attenuated their expression. Although the miR-629 mimic had no effect on the expression of ZEB1, Snail, and Slug, it enhanced the effect of TGFβ1 on N-cadherin and E-cadherin expression in ACHN cells (Fig. 6B). To further evaluate the effect of the miR-629 on EMT, we next examined its effect on TGFβ1-induced morphologic changes, motility, and invasion in ccRCC cell lines. Although TGFβ1 stimulation induced mesenchymal-like cell morphology (Fig. 6C) and upregulated cellular motility (Fig. 6D) and invasion activities (Fig. 6E), the miR-629 inhibitor marked attenuated those phenotypes in the Caki-2 cells. Inversely, the miR-629 mimic increased TGFβ1-induced cellular motility (Fig. 6F) and invasion activity (Fig. 6G) in ACHN cells. These results suggest that miR-629 could promote EMT by inducing the expression of EMT-related molecules via TGFβ in ccRCC cells. Furthermore, we evaluated the effect of TRIM33 knockdown on TGFβ1-induced cellular motility and invasion activities. TRIM33 knockdown significantly upregulated the effect of TGFβ1 on cellular motility (Supplementary Fig. S4A) but not on cellular invasion (Supplementary Fig. S4B) in ACHN cells. These results suggest that ccRCC cell motility by TGFβ1 is mediated by a miR-629–TRIM33 axis, whereas miR-629 might regulate TGFβ-

Figure 3.

miR-629 targets TRIM33 in ccRCC cells. A, predicted miR-629–binding site within the 3′-UTR of the human TRIM33 gene. The number indicates the nucleotide position in reference to the start of the predicted miR-629 binding in TRIM33 3′-UTR. Caki-2 cells and ACHN cells were cotransfected with the reporter construct containing the predicted miR-629–binding site in the TRIM33 3′-UTR and miR-629 inhibitor, or negative control (NC) miRNA inhibitor (B) or with the miR-629 mimic or negative control miRNA mimic (C). Values presented as mean ± SD of six independent experiments. **, P < 0.01 versus control inhibitor; ***, P < 0.001 versus control mimic. D and E, Caki-2 cells and ACHN cells were transfected with the miR-629 inhibitor (D) or with the miR-629 mimic (E) for 72 hours, and then protein samples were collected and subjected to Western blot analysis with anti-TRIM33 antibody. The membrane was reprobed with the anti-β-tubulin antibody. Relative TRIM33 expression is shown as mean ± SD of 4 (D) and 3 (E) independent experiments. **, P < 0.01 versus control inhibitor; ***, P < 0.001 versus control mimic. F, the expression of TRIM33 in ccRCC specimens was examined by immunohistochemical staining. One normal kidney tissue and 19 ccRCC samples were examined. Staining index of TRIM33 was compared by pathologic stages and pathologic grades. Values are median, **, P < 0.05; ***, P < 0.001.
mediated invasion through not only TRIM33 but also other miR-629–targeted factor(s) in the ccRCC cells.

**Discussion**

In the present study, we determined that miR-629 was highly expressed in ccRCC and functioned as a potent regulator of the TGFβ/Smad signaling pathway by downregulating TRIM33 to enhance the sensitivity of ccRCC to TGFβ. Several miRNAs have been already shown to regulate the TGFβ/Smad signaling by directly regulating the expression of TGFβ/Smad signaling components, such as TGFβ, TGFβ receptors, and Smads (15). To our knowledge, however, miR-629 is the first miRNA that modulates the signal intensity, but not the signal output, of the TGFβ/Smad signaling in cancer cells. It is reported that TGFβ transduces both migration and invasion signals without affecting cell proliferation signals in ccRCC cells (5). Although the miR-629 mimic upregulated the effect of TGFβ on cell motility and invasion, knockdown of TRIM33 increased only the effect of TGFβ on cell motility (Fig. 6E and F). Furthermore, the miR-629 mimic promoted cell growth in ACHN cells (Fig. 2C). Target prediction programs predicted that an miR-629–putative binding sequence exists in other genes involved in cell invasion [e.g., SOX11 (21), ephrinB1 (22), COL4A5 (23), NCAM (24), and ephB6 (25)] and cell growth [e.g., ATG5 (26), RASSF6 (27), and HEYL (28)]; therefore, miR-629 might mediate ccRCC cell invasion and growth not via TRIM33 but via other target molecules.

TRIM33 has been proposed to function as a general inhibitor of the TGFβ signaling pathway because it can mediate the degradation of Smad4 by polyubiquitylation (11) or Smad4 monoubiquitylation, making it impossible for Smad4 to bind to Smad2/3 and subsequently inducing Smad4 translocation from the nucleus to the cytosol (12). On the other hand, Agricola and colleagues (29) reported that TRIM33 facilitates the binding of the Smad2/3–Smad4 complex to the promoters of the mesendoderm regulator genes Gsc and Mixl1, leading to the differentiation of mammalian embryonic stem cells. Our experiments revealed that, although having no significant effect...
on Smad4 protein amount, the miR-629 inhibitor diminished the effect of TGFβ1 on Smad activity by inhibiting Smad2/3 and Smad4 binding in ccRCC cells. In ccRCC cells, therefore, TRIM33 might inhibit the binding of Smad4 to Smad2/3 and induces the subsequent translocation of Smad2/3 from the nucleus to the cytosol, thus leading to the repression of the TGFβ1/Smad target gene expression. Further studies are required to describe the precise mechanism(s) of how TRIM33 keeps Smad2/3 from binding with Smad4.

Early-stage renal cancers do not usually exhibit obvious symptoms and are found accidently by echographic examination. Therefore, it is necessary to identify genes that are useful as biomarkers for ccRCC in routine clinical practice. Interestingly, miR-629 is upregulated in the serum of ccRCC (30) and was also found in the circulation of prostate cancer–xenografted mice (31). Moreover, circulating miR-629 detected in plasma was reported to be a possible candidate for the diagnosis of lung cancer (32). Although the combination of several biomarkers might be needed for the diagnosis of ccRCC, the present results raise the possibility that miR-629 might be a candidate biomarker and therapeutic target molecule for ccRCC.
miR-629 promotes the TGFβ-induced effect on cell migration and invasion. A, Caki-2 cells were transfected with the miR-629 inhibitor for 24 hours and then stimulated with or without TGFβ for 48 hours. Protein samples were collected and immunoblotted with anti-N-cadherin, anti-E-cadherin, anti-Slug, anti-Snail, anti-ZEB1, and anti-TRIM33 antibodies. The membrane was reprobed with the anti-β-tubulin antibody. The results are representative of three independent experiments. B, ACHN cells were transfected with the miR-629 mimic for 24 hours and then stimulated with or without TGFβ for 48 hours. Protein samples were collected and immunoblotted with anti-N-cadherin, anti-E-cadherin, anti-Slug, anti-Snail, anti-ZEB1, and anti-TRIM33 antibodies. The membrane was reprobed with the anti-β-tubulin antibody. The results are representative of three independent experiments. C, Caki-2 cells were transfected with the miR-629 inhibitor for 24 hours and then stimulated with or without TGFβ for 48 hours. Immunofluorescence staining was performed with the anti-β-tubulin antibody and 4',6-diamidino-2-phenylindole (DAPI). The results are representative of three independent experiments. White bars, 25 μm.

Disclosure of Potential Conflicts of Interest

N. Nonomura reports receiving a commercial research grant from Takeda Pharmaceutical, Novartis Pharma, Satra Zeneca. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: K. Jingushi, K. Fujita, K. Tsujikawa
Development of methodology: K. Jingushi, H. Hase, K. Tsujikawa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Jingushi, K. Kitae, Y. Kashiwagi, W. Nakata, M. Uemura, N. Nonomura, K. Tsujikawa
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Jingushi, Y. Ueda, K. Fujita, N. Nonomura, K. Tsujikawa
Writing, review, and/or revision of the manuscript: K. Jingushi, W. Nakata, K. Fujita, M. Uemura, K. Tsujikawa

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Kitae, H. Egawa, I. Obishio, R. Kawakami, Y. Kashiwagi, Y. Tukada, T. Kobayashi, K. Tsujikawa

Study supervision: W. Nakata, M. Uemura, K. Tsujikawa

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References


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Kentaro Jingushi, Yuko Ueda, Kaori Kitae, et al.


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