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miR-181a–Twist1 pathway in the chemoresistance of tongue squamous cell carcinoma

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ABSTRACT

Although many researches have been undertaken to disclose the mechanisms of chemoresistance, the mechanisms remain unclear. The aim of this study is to elucidate the role of miR-181a–Twist1 pathway in the chemoresistance of tongue squamous cell carcinoma (TSCC). We found that cisplatin-induced chemoresistance in TSCC cell lines underwent EMT (epithelial–mesenchymal transition) and was accompanied by enhancing metastatic potential (migration and invasion *in vitro*), miR-181a downregulation and Twist1 upregulation. Functional analyses indicated that miR-181a reversed chemoresistance, inhibited EMT and metastatic potential in TSCC cells. Twist1 was confirmed as a direct miR-181a target gene by luciferase reporter gene assays. Twist1 knockdown by siRNA led to a reversal of the chemoresistance, inhibited EMT and metastatic potential in TSCC cells. Our study demonstrates that miR-181a–Twist1 pathway may play an important role in the development of cisplatin-chemoresistance, with EMT and an increase the metastatic potential of TSCC cells.

1. Introduction

Tongue squamous cell carcinoma (TSCC) is the most common carcinoma of oral squamous cell carcinoma (OSCC), with properties of rapid local invasion and metastatic spread. Cisplatin (DDP) is a commonly used chemotherapeutic agent, which is effective as a single agent or in combination with other drugs for the treatment of TSCC [1]. Treatment with cisplatin-based chemotherapy has been found to improve the prognosis of patients with TSCC [2]; however, one of the most important clinical problems for cisplatin-based chemotherapy is the intrinsic/acquired chemoresistance of cisplatin [3]. To date, many research groups have studied the various mechanisms of drug resistance, hoping to overcome this major chemotherapeutic obstacle [3–5].

Deregulation of microRNAs (miRNAs) has been observed in many tumor types, including TSCC [6]. Recently, miRNA was implicated in oncogenic cell processes, including chemoresistance [3,7–10]. In cisplatin-resistant Tca8113 cells, Yu et al. found that miR-214 and -23a increased with chemoresistance against cisplatin, while miR-21 decreased with chemosensitivity for cisplatin [3]. Pogribny et al. also reported that the expression of miR-200b was decreased in adriamycin and cisplatin-resistant MCF-7 human breast adenocarcinoma cells [8]. Recently, miR-181a was found to be related to chemoresistance in leukemia HL-60 cells [9]. According to the microarray result of Sun's research [10], miR-181a was also found to be down-regulated in cisplatin chemoresistant CAL27 cells (TSCC cell lines). In this study we will further confirm the role of miR-181a in the cisplatin chemoresistance of TSCC.

Recently, a series of studies also indicated that epithelial–mesenchymal transition (EMT) is involved in drug resistance in cancer cells [4,11]. Although cancer cells undergoing EMT develop resistance to anticancer agents, EMT can also be induced by anticancer agents [12,13]. In pancreatic and ovarian cancer, cancer cells resistant to gemcitabine and paclitaxel undergo EMT with increased expression of Snail and Twist1 [13,14].

Although many researches have been undertaken to disclose the mechanisms of chemoresistance, the mechanisms remain unclear. In this study, we found that chemoresistance can induce EMT in TSCC cell lines and was accompanied by enhancing metastatic potential, miR-181a downregulation and Twist1 upregulation. Functional analyses indicated that miR-181a reversed chemoresistance, inhibited EMT and the metastatic potential of TSCC cells. We also identified Twist1 as a direct target of miR-181a. Knockdown of Twist1 led to a reversal of the chemoresistance, inhibited EMT and metastatic potential in TSCC cells. Our data demonstrated that the miR-181a–Twist1 pathway may play an important role in the development of cisplatin-chemoresistance, with EMT and an increase the metastatic potential of TSCC cells.

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2. Materials and methods

2.1. Cell culture

The human tongue cancer cell lines CAL27 and SCC15 were purchased from the American Type Culture Collection (Manassas, VA, USA). The stable cisplatin-resistant cell lines (DDP-resistant CAL27 cell line [10] and DDP-resistant SCC15 cell line [15]) were kindly presented by Jingsong Li and Bin Zhang, respectively. The TSCC cells were maintained in DMEM/F-12 (Gibco, CA, USA) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2. For functional analyses, the miR-181a mimic, control mimic, specific locked nucleic acid (LNA) inhibitor for miR-181a (miR-181a LNA), control LNA, Twist1 siRNA and control non-targeting siRNA (Genepharma, Shanghai, CHN) were transfected into the appropriate cells using Lipofectamine Transfection Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, as previously described [16]. The sequences of the miRNA and siRNA for transfection were shown in Supplementary Table S1.

2.2. Cisplatin chemosensitivity assay

Cells were plated in triplicate in 96-well plates at a density of 5000 cells. After 24 h incubation, the medium was replaced by fresh medium with or without various concentrations of cisplatin (Sigma–Aldrich, MO, USA). Then cell viability was assayed 48 h later using a modified MTT assay as our previously described [17]. IC_{50} values were defined as the concentrations resulting in a 50% reduction in growth compared to control cell growth.

2.3. Immunofluorescence staining

Immunofluorescence staining was performed as our previously described [18]. Briefly, the cells were cultured on 22-mm coverslips in 6-well plates and then fixed in 4% paraformaldehyde. Then, the cells were incubated with primary antibodies against E-cadherin and Vimentin (Santa Cruz, CA, USA), followed by incubation with the secondary antibody. The slides were examined using a fluorescence microscope (Olympus). The red fluorescence shows the localization of E-cadherin and Vimentin, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Representative images of the cells were captured at a magnification of 400×.

2.4. Western blots

Western blots were performed as our previously described, using antibodies specific against E-cadherin, Vimentin, Twist1 and GAPDH (Santa Cruz, CA, USA) [16]. GAPDH, as loading control, was done at the same blots after stripping of the membrane.

2.5. Real-time PCR analysis

The relative expression level of miR-181a was determined by real-time PCR as our previously described [17]. The miR-181a expression level was normalized with U6. The primers for miR-181a were as follows: F: 5′-gcugucggugagctcaactggtgtcgtgg agtcggcaattcagttgagactcaccg-3′; R: 5′-acactccagctgggaacattcaacgctgtcg-3′.

2.6. Wound healing assay

Cell migration ability was measured using a wound healing assay as our previously described [16]. Briefly, the cells were grown to confluence and "wounded" by dragging a 1 ml pipette tip. Images were taken at time points 0 and 24 h post-wounding using a Nikon Diaphot TMD inverted microscope (40×). The relative distance traveled by the leading edge from 0 to 24 h was assessed. The more relative distance traveled mean the higher migration ability.

2.7. Transwell invasion assay

Cell invasion ability was measured using transwell invasion assays as our previously described [16]. Briefly, cells were seeded in serum-free medium in the upper chamber (Biocoat Matrigel invasion chamber, BD Biosciences, NJ, USA) and allowed to invade through the Matrigel to the lower chamber for 24 h. The cells on the bottom surface of the filter were rinsed and permeabilized with 1% Triton X-100, stained with crystal violet and visualized under a microscope. Three random fields were captured at 200× magnification ($n = 3$). The number of cells on the bottom surface was compared between groups.

2.8. miRNA target prediction and dual luciferase reporter assay

Based on miRNA target prediction tools (microRNA.org), Twist1 genes were predicted to be candidate targets of miR-181a. To verify this prediction, luciferase reporter assays were executed as our previously described [19]. The 3′-untranslated region (3′-UTR) of Twist1 containing the miR-181a binding sites was synthesized using PCR [F: 5′-ccgctcgagctagatgtcattgtttccagag-3′; R: 5′-ataagaatgcggccgctgcatttagacaccggatc-3′]. The resulting PCR product was subcloned into the XhoI–NotI site of the luciferase reporter vector psiCHECK™-2 (Promega) (p-Twist1). The corresponding mutant constructs were created by mutating the seed regions of the miR-181a binding sites [F: 5′-gtttgtgttttttttttttttttttttgacgaacttacattttattttttattttttcatgcatgcattctc-3′; R: 5′-gagaatgcatgcatgaaaaaaataaaaataaaatgtaagttcgtcaaaaaaaaaaaaaaaaaaaacacaaac-3′], (p-Twist1 mut). These constructs were verified by sequencing. Then, 293 T cells were co-transfected with miR-181a mimic (or miR-181a LNA) and p-Twist1 (or its mutants). After 48 h, the transfected cells were harvested with ice-cold PBS, and dual luciferase assays were performed according to the manufacturer's protocol.

2.9. Statistical analysis

All experiments were performed in triplicate and data are presented as mean ± SD. The data were analyzed using Student's *t*-test to determine significance between two variables. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. Cisplatin chemoresistance induced EMT, enhanced metastatic potential and down-regulated miR-181a in TSCC cells

The cisplatin chemoresistance of TSCC cells (CAL27 cell line) was confirmed as shown in Fig. 1A. The IC_{50} of DDP-resistant CAL27 cells were 7.5-fold higher compared to the parental cell lines CAL27 ($P < 0.01$). A higher IC_{50} value was considered to correspond to clinical cisplatin resistance.

To investigate whether chemoresistance induced EMT, morphologic changes and EMT markers were evaluated. As shown in Fig. 1B, CAL27 cells had a typical epithelial cobblestone appearance, tight cell–cell junctions and grow in clusters, but the DDP-resistant CAL27 cells displayed an irregular fibroblast-like morphology and were elongated and separated from each other. Both Western blotting and immunofluorescence staining demonstrated that the epithelial marker E-cadherin was down-regulated and the mesenchymal marker Vimentin was up-regulated in

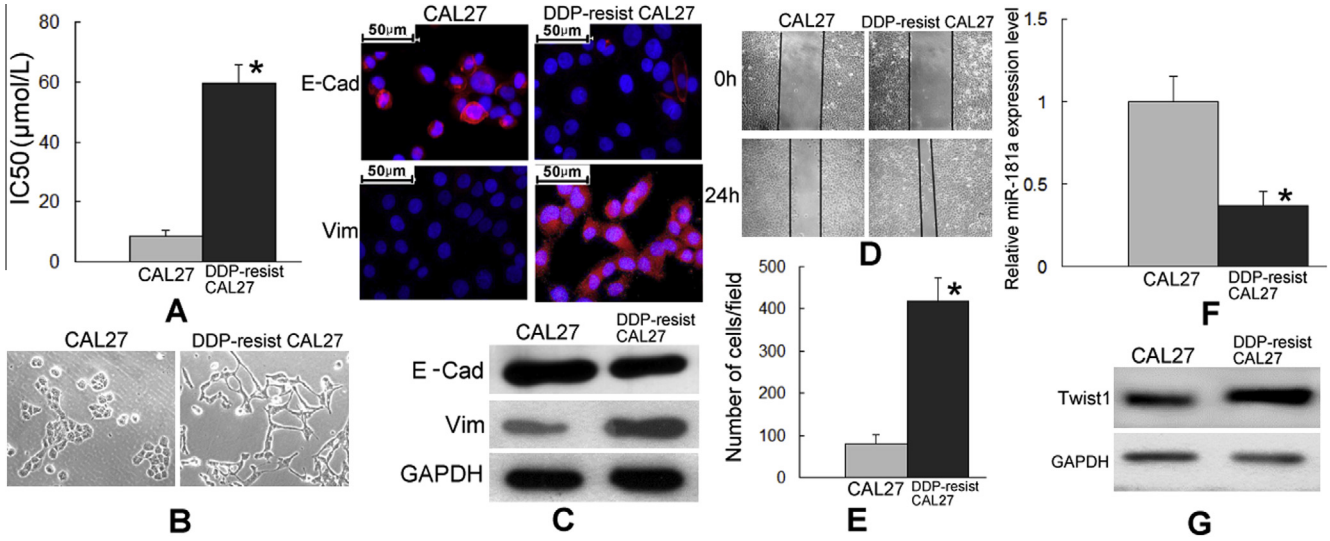

Fig. 1. Cisplatin chemoresistance induced EMT, enhanced metastatic potential and down-regulated miR-181a in tongue cancer cells. (A) The IC_{50} values of DDP-resistant CAL27 cells were significantly higher than that of CAL27 cells $^{*}P < 0.01$. (B) The morphology showed that CAL27 cells had a typical epithelial cobblestone appearance, while DDP-resistant CAL27 cells displayed irregular fibroblast-like morphology (200×). (C) Both Western blotting and immunofluorescence staining demonstrated that E-cadherin was down-regulated and Vimentin was up-regulated in DDP-resistant CAL27 cells compared to CAL27 cells, scale bar: 50 μm. (D and E) The migratory abilities (D, wound healing assay) and invasive abilities (E, transwell invasion assay) of DDP-resistant CAL27 cells were higher than that of CAL27 cells $^{*}P < 0.01$. (F and G) The expression of miR-181a was down-regulated (F) and the expression of Twist1 was up-regulated (G) in DDP-resistant CAL27 cells compared to CAL27 cells $^{*}P < 0.01$.

DDP-resistant CAL27 cells than in CAL27 cells (Fig. 1C). The metastatic potential of cisplatin chemoresistant TSCC cells were examined by wound healing and transwell invasion assays. As shown in Fig. 1D and E, DDP-resistant CAL27 cells exhibited faster cell migration and significantly elevated invasions ($P < 0.01$) as compared to their paired cell lines, CAL27 cells.

Moreover, we found that the expression of miR-181a was significantly down-regulated ($P < 0.01$) and the expression of Twist1 was up-regulated in DDP-chemoresistant CAL27 cells compared to their parental lines CAL27 cells (shown in Fig. 1F and G). In another TSCC cell line (SCC15 cell line), we also found that DDP-resistant SCC15 cells induced EMT, enhanced metastatic potential, down-regulated miR-181a and up-regulated Twist1 as compared to their parental lines SCC15 cells (shown in Supplementary Fig. S1A–G). Taken together, these results indicated that cisplatin chemoresistance induced EMT, enhanced the metastatic potential and down-regulated miR-181a in TSCC cells.

3.2. miR-181a reversed chemoresistance, inhibited EMT and metastatic potential in TSCC cells

To investigate of the role of miR-181a in the chemoresistance of TSCC cells, miR-181a mimics and miR-181a LNA were transfected into DDP-resistant CAL27 (Fig. 2A) or CAL27 (Fig. 3A), respectively. In the DDP-resistant CAL27 cells, transfection with miR-181a mimics significantly decreased the value of IC_{50} as compared to the control mimics transfection (Fig. 2B, $P < 0.01$). As compared to transfection with control mimics, transfection with miR-181a mimics in DDP-resistant CAL27 cells showed obvious morphologic changes, from scattered, spindle shapes to tightly packed cobblestone shapes (Fig. 2C), as well as E-cadherin upregulation and Vimentin downregulation (Fig. 2D). In addition, transfection with miR-181a mimics suppressed the metastatic potential of DDP-resistant CAL27 cells, exhibited slower cell migration and significantly decreased invasions ($P < 0.01$) as compared to transfection with control mimics (Fig. 2E and F). On the other hand, transfection of miR-181a LNA into CAL27 cells significantly increased the value of IC_{50} compared to the control LNA transfection (Fig. 3B, $P < 0.01$). As compared to transfection with control LNA, Transfection with miR-181a LNA in CAL27 cells induced morphologic changes consistent with EMT, with the cells displaying irregular fibroblast-like morphology, elongated and separated from each other (Fig. 3C), and accompanied with E-cadherin downregulation and Vimentin upregulation (Fig. 3D). Transfection with miR-181a LNA also enhanced the metastatic potential of CAL27 cells, exhibited faster cell migration and significantly elevated invasions ($P < 0.01$) as compared to transfection with control LNA (Fig. 3E and F). Thus, these results revealed that miR-181a reversed chemoresistance, inhibited EMT and metastatic potential in TSCC cells.

3.3. miR-181a directly targeted Twist1 and then reversed chemoresistance and EMT in TSCC cells

Based on the bioinformatics analysis, a conserved miR-181a targeting sequence was identified in the 3′-UTR of Twist1 mRNA (Supplementary Fig. S2). To confirm that miR-181a directly targeted this sequence, dual-luciferase reporter assays were performed. As illustrated in Fig. 4A, when cells were co-transfected with p-Twist1 and miR-181a mimic, luciferase activity was significantly reduced compared to cells co-transfected with p-Twist1 and control mimic ($P < 0.05$). When cells were treated with p-Twist1 and miR-181a LNA, the luciferase activity was significantly enhanced compared to the cells treated with p-Twist1 and control LNA (Fig. 4B, $P < 0.05$). Furthermore, when the seed region of the targeting site was mutated (p-Twist1 mut), the effect on the miR-181a luciferase activity was abolished (Fig. 4A and B). Moreover, the expression of Twist1 was down-regulated in miR-181a mimic transfected DDP-resistant CAL27 cells and up-regulated in miR-181a LNA transfected CAL27 cells compared to control mimics transfection or control LNA transfection, respectively (Fig. 4C). Therefore, our study demonstrated that miR-181a directly targeted Twist1 in TSCC cells.

To further evaluate the role of Twist1 in chemoresistance of TSCC cells, we analyzed the effect of Twist1 knockdown in DDP-resistant CAL27 cells (Fig. 4C). As shown in Fig. 4D, knockdown of Twist1 in DDP-resistant CAL27 cells significantly decreased the value of IC_{50} compared to the control siRNA transfection

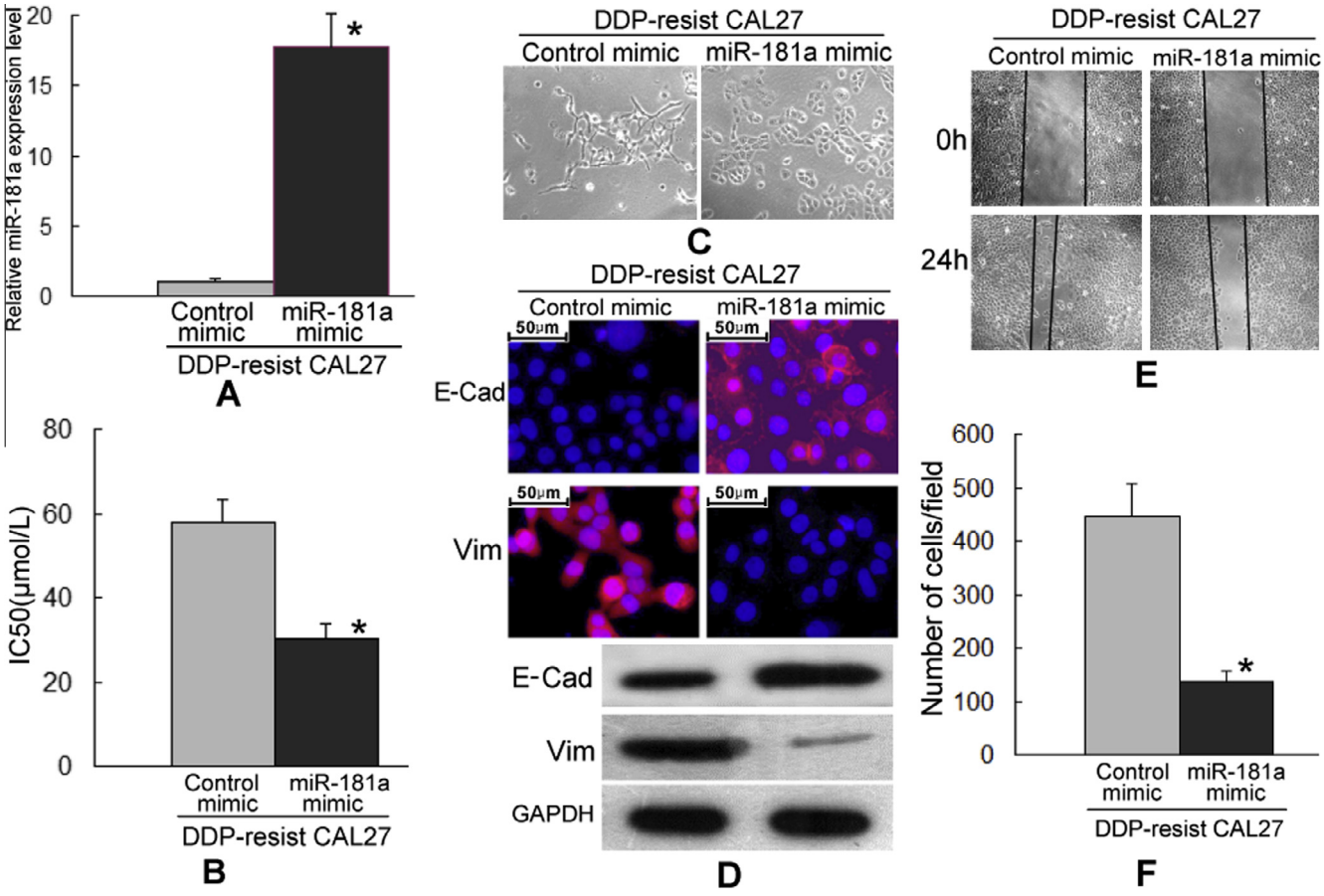

Fig. 2. miR-181a mimic reversed chemoresistance and inhibited EMT in DDP-resistant CAL27 cells. (A) The expression of miR-181a was significantly increased in DDP-resistant CAL27 cells after transfected with miR-181a mimics $^{*}P < 0.01$. (B) Transfection with miR-181a mimics significantly decreased the IC_{50} of DDP-resistant CAL27 cells as compared to control mimics transfection $^{*}P < 0.01$. (C and D)Transfection with miR-181a mimics in DDP-resistant CAL27 cells was associated with an obvious morphologic change, from scattered, spindle shapes to tightly packed cobblestone shapes (200×, C); up-regulated the expression of E-cadherin and down-regulated the expression of Vimentin as compared to control mimics transfection (D), scale bar: 50 μm. (E and F) Transfection with miR-181a mimics suppressed the migration and invasion of DDP-resistant CAL27 cells as compared to control mimics transfection, as shown by the wound healing assay (E) and the transwell invasion assay (F) $^{*}P < 0.01$.

($P < 0.05$). Moreover, as compared to transfection with control siRNA, transfection with Twist1 siRNA in DDP-resistant CAL27 cells led to obvious morphologic changes, from scattered, spindle shapes to tightly packed cobblestone shapes, as well as E-cadherin upregulation and Vimentin downregulation (Supplementary Fig. S3A and B). Twist1 siRNA suppressed the metastatic potential of DDP-resistant CAL27 cells, exhibited slower cell migration and significantly decreased invasions ($P < 0.01$) as compared to transfection with control siRNA (Supplementary Fig. 3C and 2D). Thus, these results demonstrated that Twist1 siRNA led to a reversal of chemoresistance, inhibited EMT and metastatatic potential in TSCC cells.

4. Discussions

Although cisplatin-based chemotherapy has been an effective method for the treatment of tongue cancer, chemoresistance has become a major chemotherapeutic obstacle. Recently, a series of studies indicated that EMT is involved in drug-resistance in cancer cells. Many chemotherapeutic agents were shown to induce EMT in several types of cancers [12,13,20–24], including head and neck carcinoma [21], hepatocellular carcinoma [22], breast cancer [23], pancreatic and ovarian cancers [12,13]. On the other hand, EMT was shown to enhance the chemoresistance of the tumor cells in pancreatic cancer, breast cancer, colorectal cancer and ovarian cancer [4,11,23,25]. The above studies provide strong evidence linking chemoresistance to EMT. In the present study, we found that chemoresistant TSCC cells undergo EMT program. Moreover, we found that DDP-resistant TSCC cells in TSCC cells were accompanied with enhancing metastatic potential, miR-181a downregulation and Twist1 upregulation.

A number of dysregulated miRNAs have been implicated either as oncogenes or tumor suppressors, affecting the initiation and progression of OSCC through the regulation of proliferation, apoptosis and metastasis [6,19]. Shin et al. found that miR-181a was frequently downregulated in OSCC, ectopic expression of miR-181a suppressed proliferation and anchorage independent growth ability of OSCC [26]. However, Yang et al. found that over-expression of miR-181 was correlated with lymph-node metastasis and a poor survival, functional assays revealed ectopically over-expressed miR-181 would enhance cell migration and invasion of OSCC cells [27]. In our previous report, we found that miR-181a was down-regulated in higher metastatic salivary adenoid cystic carcinoma cell lines (SACC-LM cell line), functional analysis indicated that miR-181a inhibited SACC cell migration, invasion and proliferation *in vitro*, and suppressed tumor growth and lung metastasis in vivo [28]. In the present study, we also found that miR-181a was down-regulated in higher metastatic TSCC cells.

Recently, researches have found that miRNAs contribute to chemoresistance and modulation of EMT [7,29,30]. For example, members of the miR-200 miRNA family are down-regulated in

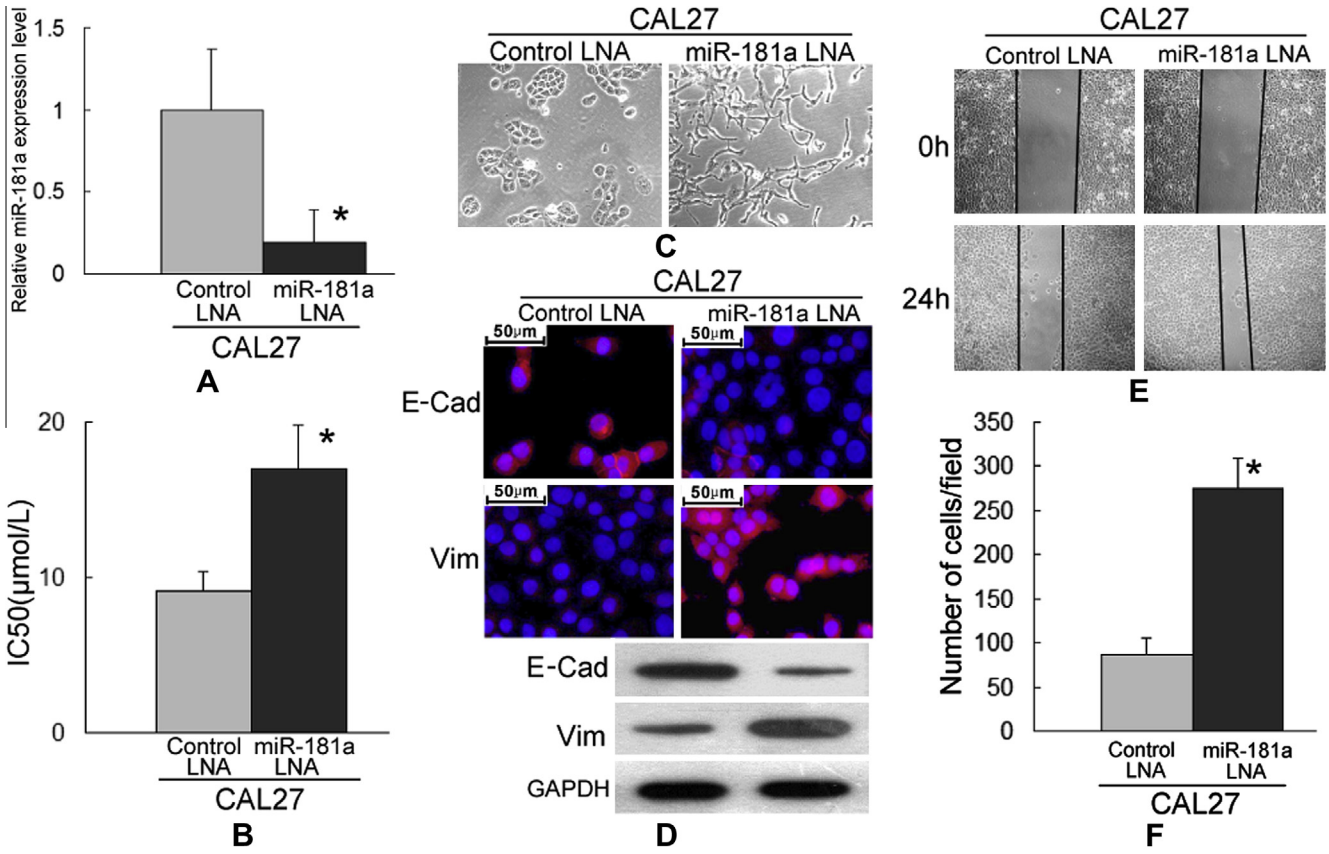

Fig. 3. miR-181a LNA induced chemoresistance and EMT in CAL27 cells. (A) The expression of miR-181a was significantly decreased in CAL27 cells after transfected with miR-181a LNA $^{*}P < 0.01$. (B) Transfection with miR-181a LNA significantly increased the IC_{50} of CAL27 cells as compared to control LNA transfection $^{*}P < 0.01$. (C and D) Transfection with miR-181a LNA in CAL27 cells was associated with an obvious morphologic change, from tightly packed cobblestone shapes to scattered, spindle shapes (200×, C); down-regulated the expression of E-cadherin and up-regulated the expression of Vimentin as compared to control LNA transfection (D), scale bar: 50 μm. (E and F) Transfection with miR-181a LNA suppressed the migration and invasion of CAL27 cells as compared to control LNA transfection, as shown by the wound healing assay (E) and the transwell assay (F) $^{*}P < 0.01$.

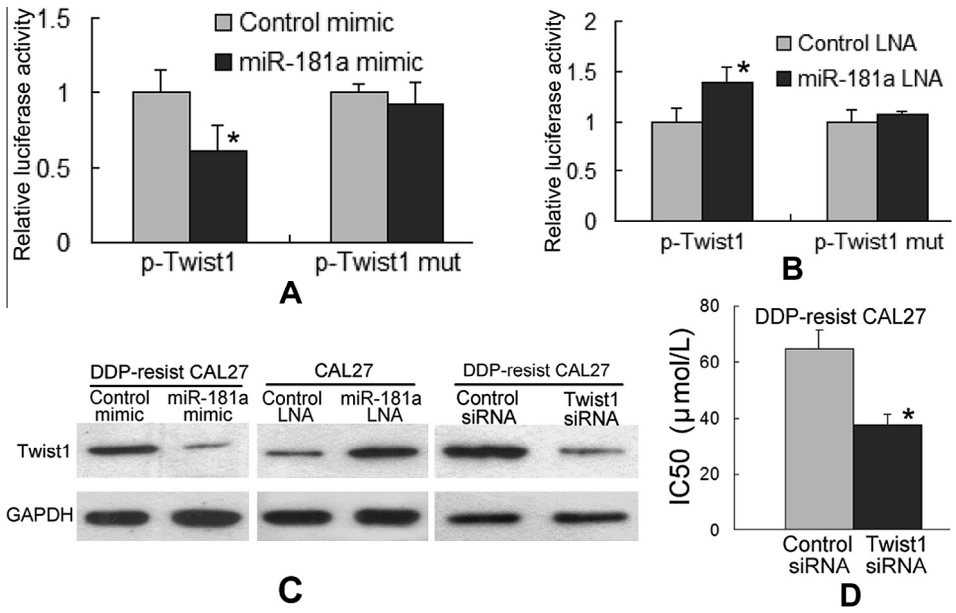

Fig. 4. miR-181a directly targeted Twist1 and then reversed chemoresistance in TSCC cells. (A and B) Dual luciferase reporter assays were performed to test the miR-181a target gene. When cells were transfected with p-Twist1, the luciferase activity was significantly reduced in the miR-181a mimic-treated cells but significantly enhanced in the miR-181a LNA-treated cells. When the seed region of the targeting site was mutated (p-Twist1 mut), the effects of miR-181a on luciferase activity were abolished $^{*}P < 0.05$. (C) miR-181a mimic transfection reduced Twist1 protein levels in DDP-resistant CAL27 cells as compared to control mimic transfection, while the expression of Twist1 was up-regulated in miR-181a LNA transfected CAL27 cells as compared to control LNA transfection. The expression of Twist1 was down-regulated in DDP-resistant CAL27 cells after transfected with Twist1 siRNA as compared to control siRNA transfection. (D) Twist1 knockdown in DDP-resistant CAL27 cells decreased the values of IC_{50} as compared to control siRNA transfection ($^{*}P < 0.05$).

many human cancer cells and play a critical role in the suppression of EMT, chemoresistance and metastasis [30]. Until now, few studies have been published investigating miR-181a and chemoresistance, and miR-181a and EMT. Bai et al. revealed that miR-181a expression was downregulation in the Ara-C-resistant HL-60 cells compared with its parental cells HL-60. Over-expression of miR-181a in HL-60/Ara-C cells sensitized the cells to Ara-C treatment [9]. In the present study, we found that the expression level of miR-181a was downregulation in DDP-resistant TSCC cells. miR-181a mimic transfection in DDP-resistant TSCC cells sensitized the cells to DDP treatment, reversed EMT and inhibited migration and invasion. These results mean that miR-181a played an important role in chemoresistance, EMT and metastatic potential in TSCC cells.

It had been reported that the precise regulation of the diversified biological processes of miR-181a was dependent on the ability to regulate multiple target genes, such as K-ras, Bcl-2 (B-cell lymphoma 2), and ATM (Ataxia telangiectasia mutated) [26]. In our previous report, we had reported that MAP2K1, MAPK1 and Snai2 were direct target gene of miR-181a [28]. In the present study, we also confirmed that miR-181a directly targeted the 3′-UTR of Twist1 mRNA. Previous researches had suggested that Twist1 plays a critical role in chemoresistance and EMT [31,32]. In the present study, Twist1 was found to be downregulation in higher metastatic DDP-resistant TSCC cells. Upregulation of miR-181a resulted in decreasing expression of Twist1. Knockdown of Twist1 reversed the chemoresistance and EMT, inhibited the migration and invasion of TSCC cells. These results suggested that miR-181a regulated chemoresistance, EMT, migration and invasion in TSCC cells by targeting Twist1 mRNA.

From above we demonstrated that chemoresistance can induce EMT in TSCC cell lines and was accompanied with higher metastatic potential, miR-181a downregulation and Twist1 upregulation. A functional analysis indicated that miR-181a played an important role in the process of chemoresistance, EMT and metastasis in TSCC cells. Furthermore, we identified Twist1 as a direct target of miR-181a. Knockdown of Twist1 led to a reversal of the chemoresistance, inhibited EMT and metastatic potential in TSCC cells. Thus, our data elucidated that the miR-181a–Twist1 pathway may play an important role in the development of cisplatin-chemoresistance, with EMT and an increase the metastatic potential of TSCC cells.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.051.

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