miR-23a promotes cisplatin chemoresistance and protects against cisplatin-induced apoptosis in tongue squamous cell carcinoma cells through Twist

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Abstract. Tongue squamous cell carcinoma (TSCC) is one of the most common head and neck cancers. Cisplatin is effective as a single agent or in combination with other drugs for the treatment of TSCC. Treatment with cisplatin-based chemotherapy has been found to improve the prognosis of patients with TSCC. However, one of the most important clinical issues of cisplatin-based TSCC chemotherapy is the intrinsic/acquired chemoresistance to cisplatin. Increased expression of miR-23a reportedly promotes cisplatin chemoresistance in TSCC cells. High expression of Twist is also associated with cancer chemoresistance and poor prognosis of TSCC patients. In the present study, we explored the interaction between miR-23a and Twist in TSCC cells, and assessed its impact on TSCC chemoresistance to cisplatin. miR-23a and/or Twist were overexpressed or knocked down in SCC-4 and Tca8113 human TSCC cells. The expression levels of miR-23a and Twist were determined. The half maximal inhibitory concentration (IC_{50}) of cisplatin and cell apoptosis rate under cisplatin treatment were used as measures of cisplatin chemoresistance. Overexpression of miR-23a in both SCC-4 and Tca8113 cells markedly increased Twist expression, c-Jun N-terminal kinase (JNK) activity and the half maximal inhibitory concentration (IC_{50}) of cisplain, and decreased cisplatin-induced apoptosis, all of which was abolished by knockdown of Twist or selective JNK inhibitor SP600125. On the other hand, knockdown of miR-23a significantly decreased Twist expression, JNK activity and IC_{50} of cisplain, and increased cisplatin-induced apoptosis, all of which was completely reversed by overexpression of Twist. In conclusion, the present study for the first time demonstrates that miR-23a promotes cisplatin chemoresistance and protects against

cisplatin-induced apoptosis in TSCC cells through inducing Twist expression by a JNK-dependent mechanism. It adds new insights into the molecular mechanisms underlying TSCC chemoresistance.

Introduction

Oral squamous cell carcinoma is the most common head and neck cancer. It accounts for more than 90% of all head and neck cancers and has a poor prognosis (1). Tongue squamous cell carcinoma (TSCC) is the most common oral squamous cell carcinoma. It is associated with poorer survival and a lower rate of local tumor control than other sites of head and neck cancer, with a 5-year survival rate of 50% (2). Chemotherapy has been widely accepted as one of the three major therapies (surgery, chemotherapy and radiotherapy) treating advanced squamous cell carcinoma in the head and neck region (3). Cisplatin, a commonly used chemotherapeutic agent, is effective as a single agent or in combination with other drugs for the treatment of TSCC (4). Treatment with cisplatin-based chemotherapy has been found to improve the prognosis of patients with TSCC (5). However, one of the most important clinical issues of cisplatin-based TSCC chemotherapy is the intrinsic/acquired chemoresistance to cisplatin (6).

Traditionally, the targets and modulators of chemotherapy most in focus are DNA, mRNA and proteins (3). Recently, it has been found that microRNAs (miRNAs) are implicated in oncogenic cell processes including chemoresistance (7). miRNAs are small non-coding RNA molecules of 19-25 nucleotides in length. They regulate target gene expression post-transcriptionally by incomplete base pairing with their target mRNAs (8). They function through RNA-induced silencing complexes, targeting them to mRNAs where they either repress translation or direct destructive cleavage (9). A recent study has shown that the increased expression of miR-23a promotes cisplatin chemoresistance in TSCC cells (3).

Wist, also known as Twist1, belongs to the basic helix-loop-helix transcription factor family. High expression of Twist has been detected in several types of cancers and has been associated with cancer progression and chemoresistance (10). A recent study has shown that overexpression of Twist is associated with

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poor prognosis of TSCC patients (11), which suggests that Twist could be a potential therapeutic target for TSCC.

Our pilot study suggested that miR-23a regulates the expression of Twist in TSCC cells. In the present study, we explored the interaction between miR-23a and Twist in TSCC cells, and assessed its impact on TSCC chemoresistance to cisplatin.

Materials and methods

Cell lines, plasmids and reagents. Human TSCC cell lines SCC-4 and Tca8113 were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA) and the Wuhan Boster Biological Engineering Inc. (Wuhan, China), respectively. Twist (sc-38604-V) shRNA lentiviral particles, control shRNA lentiviral particles-A (sc-108080) and anti-Twist (sc-81417) and anti-β-actin (ACTBD11B7) (sc-81178) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SuperFect transfection reagent was purchased from Qiagen (Valencia, CA, USA). The non-radioactive c-Jun N-terminal kinase (JNK) assay kit (#8794) was purchased from New England Biolabs (Beverly, MA, USA). Human Twist cDNA was subcloned into the pcDNA 3.1 expression vector (12). The pMIRH23aPA-1 lentiviral vector expressing miR-23a, the pMIRZIP-23a lentiviral vector expressing shRNA against miR-23a, and the lentiviral packaging system were purchased from System Biosciences (Mountain View, CA, USA). The Annexin V-EGFP apoptosis detection kit was purchased from BioVision (Mountain View, CA, USA). Puromycin, cisplatin, selective JNK inhibitor SP600125 and all chemicals of reagent grade were purchased from Sigma (St. Louis, MO, USA).

Transfection and lentiviral transduction. The Twist expression vector was transfected into cells using SuperFect transfection reagent according to the manufacturer's instructions. Pools of stable transductants were generated via selection with G418 (600 μg/ml) by the manufacturer's protocol. Lentiviral transduction of Twist-shRNA was performed in SCC-4 and Tca8113 cells. Pools of stable transductants were generated via selection with puromycin (4 μg/ml) according to the manufacturer's protocol (Santa Cruz Biotechnology). SCC-4 and Tca8113 cells were also transduced with the lentivirus expressing miR-23a (pMIRH23aPA-1) sequences, shRNAs against miR-23a (pMIRZIP-23a), or control scrambled hairpin vector sequences under the control of constitutive H1 promoter (Systems Biosciences). Lentiviral supernatants were used to infect the cells with the addition of Polybrene at 8 μg/ml for 8 h. Cells were harvested 48 h after the lentiviral transduction.

Real-time quantitative reverse transcription PCR. Total RNA was prepared from cells using TRIzol reagent followed by purification with TURBO DNA-free system (Ambion, Austin, TX, USA). The cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed using SYBR-Green PCR Master Mix in a 7300 Real-Time PCR system (both from Applied Biosystems, Foster City, CA, USA). TaqMan microRNA assays (Applied Biosystems) that include RT primers and TaqMan probes were used to quantify the

expression of miRNA-23a; RNU66 (PN 4373382) was used as an internal control (Applied Biosystems) for determining the expression level of miR-23a. For measurement of the Twist mRNA level, the following primers were used: for Twist, 5'-AC GAGCTGGACTCCAAGATG-3' (forward) and 5'-CACGCC CTGTTTCTTTGAAT-3' (reverse); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GACTCATGACCACAGT CCATGC-3' (forward) and 5'-AGAGGCAGGGATGATGTT CTG-3' (reverse). The results were normalized against that of the GAPDH gene in the same sample. Each experiment was repeated for three times in duplicates.

Western blot analysis. Briefly, cells were dissolved in 250 μl of 2X SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromphenol blue, 5% 2-mercaptoethanol), and incubated at 95˚C for 10 min. Equal amount of proteins for each sample were separated by 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). Membranes were incubated for 1 h with a 1/500 dilution of primary antibody, and then washed and revealed using secondary antibodies conjugated with horseradish peroxidase (1/5,000; 1 h). Peroxidase was revealed with a GE Healthcare ECL kit (Shanghai, China).

JNK activity assay. The JNK activity was measured with a non-radioactive assay kit as per the manufacturer's protocol (New England Biolabs) (13). Briefly, JNK was precipitated from the cell lysate by c-Jun fusion protein bound to glutathione sepharose beads. c-Jun contains a high affinity-binding site for JNK, N-terminal to the two phosphorylation sites, Ser63 and Ser73. After selectively pulling down JNK using c-jun fusion protein beads, the beads were extensively washed and the kinase reaction was carried out in the presence of cold ATP in a final volume of 25 μl. The reaction was stopped with 25 μl of 2X SDS sample buffer and loaded onto a 10% polyacrylamide gel. Protein was transferred to nitrocellulose by electroblotting, and c-jun phosphorylation was selectively measured using the phospho-c-Jun antibody.

Cisplatin chemosensitivity/chemoresistance assay. Cells were plated in duplicates in 96-well plates at a density of 5,000 cells. After 24 h of incubation, the medium was replaced by fresh medium with or without various concentrations of cisplatin (Sigma). Then cell viability was assayed 48 h later using a modified MTT assay as previously described (14). The half maximal inhibitory concentration (IC_{50}) was defined as the concentration resulting in a 50% reduction in growth compared to control cells.

Apoptosis analysis. Cells were seeded at $5x10^5$/well in 6-well plates. After cells attached to the plates (~2 h after seeding), cells were treated with 20 μM of cisplatin for 30 h. Cell apoptosis was analyzed with an Annexin V-EGFP apoptosis detection kit coupled with flow cytometric analysis according to the manufacturer's instructions (BioVision).

Statistical analysis. Statistical analyses were performed with SPSS for Windows 10.0 (IBM, Chicago, IL, USA). All data values are expressed as means ± SD. Comparisons of means among multiple groups were performed with one-way ANOVA
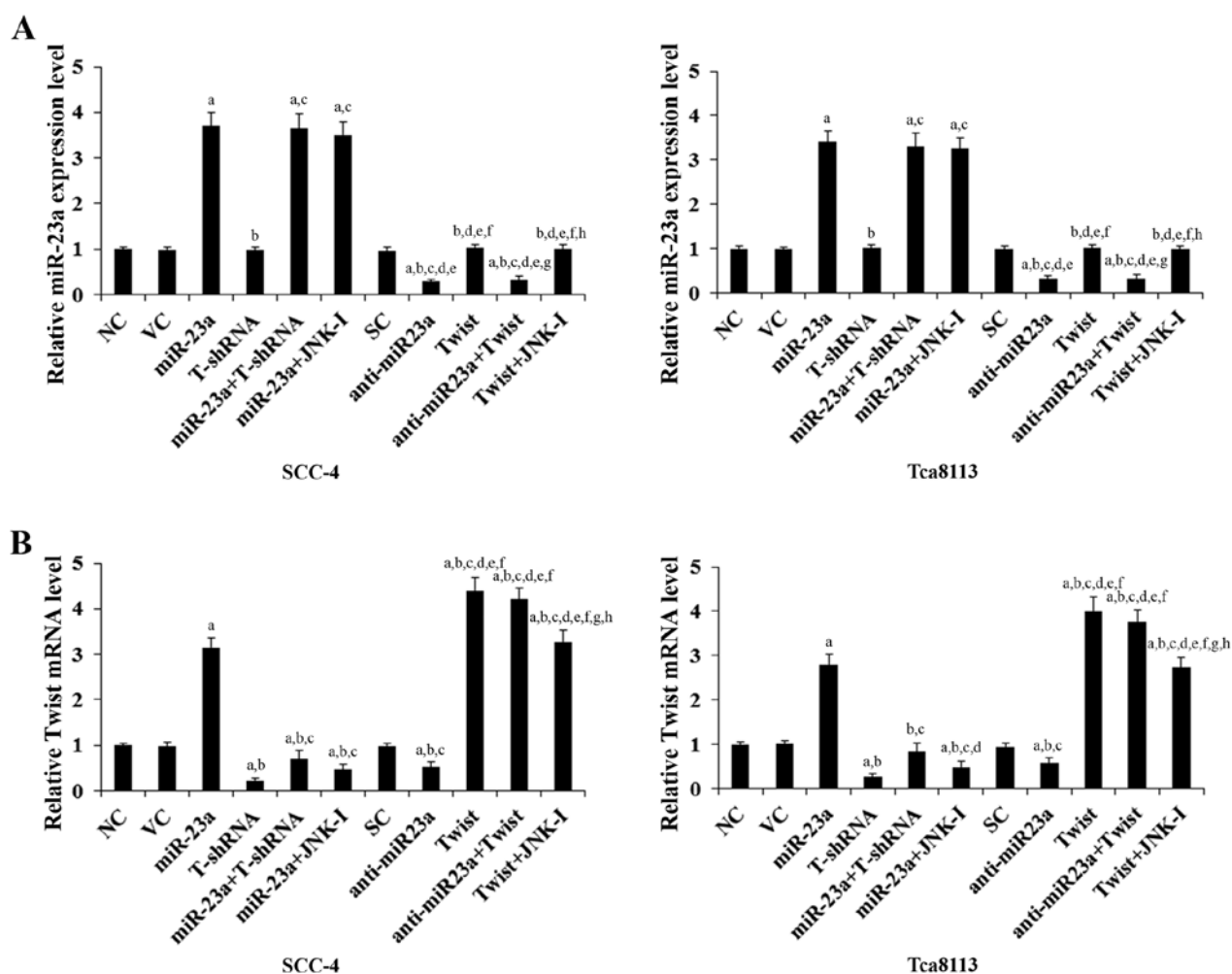
Figure 1. Expression levels of miR-23a and Twist mRNA in human tongue squamous cell carcinoma (TSCC) cells with overexpression and knockdown of miR-23a and/or Twist. In SCC-4 and Tca8113 human TSCC cells, the expression levels of (A) miR-23a and (B) Twist mRNA were determined by real-time quantitative reverse transcription PCR in normal control cells (NC), cells transduced with empty lentiviral vector pMIR and scramble control lentiviral shRNA (VC), cells overexpressing miR-23a (transduced with lentiviral pMIRH23aPA-1), cells stably transduced with lentiviral Twist-shRNA (T-shRNA), cells stably transduced with lentiviral Twist-shRNA and overexpressing miR-23a (miR-23a+T-shRNA), cells overexpressing miR-23a and treated with selective c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10 μM) for 30 min (miR-23a+JNK-I), cells transduced with lentiviral vector pMIRZIP expressing scramble control hairpin vector sequences and transfected with empty pcDNA3.1 vector (SC), cells overexpressing lentiviral shRNA against miR-23a (anti-miR23a; transduced with lentiviral pMIRZIP-23a), cells overexpressing Twist (stably transfected with pcDNA3.1-Twist expression vector), cells overexpressing anti-miR23a and Twist, and cells overexpressing Twist and treated with SP600125 (10 μM) for 30 min (Twist+JNK-I). The expression level of miR-23a and Twist mRNA is shown as fold-changes to that of NC (designated as 1). Each experiment was repeated three times in duplicates. Data values are expressed as means ± SD. [a]$p<0.05$ vs. controls (NC, VC and SC); [b]$p<0.05$ vs. miR-23a; [c]$p<0.05$ vs. T-shRNA; [d]$p<0.05$ vs. miR-23a+T-shRNA; [e]$p<0.05$ vs. miR-23a+JNK-I; [f]$p<0.05$ vs. anti-miR23a; [g]$p<0.05$ vs. Twist; [h]$p<0.05$ vs. anti-miR23a+Twist.

followed by *post-hoc* pairwise comparisons using Tukey's test. A two-tailed $p<0.05$ was considered to indicate a statistically significant result in the present study.

Results

Overexpression and knockdown of miR-23a and Twist in human TSCC cells. To investigate the functional interaction between miR-23a and Twist in TSCC cells, we overexpressed miR-23a and Twist in SCC-4 and Tca8113 human TSCC cells, and on the other hand transduced the cells with lentiviral shRNAs to knock down miR-23a and Twist, respectively. Real-time reverse transcription PCR showed that compared with the controls, miR-23a was overexpressed by ~3.5-fold and knocked down ~70% in SCC-4 and Tca8113 cells, respectively; the Twist mRNA was overexpressed by ~4-fold and knocked

down ~75% in SCC-4 and Tca8113 cells, respectively (Fig. 1). Twist expression at both the mRNA (Fig. 1) and protein levels (Fig. 2) in the TSCC cells increased (by ~3-fold at the mRNA and 2.6-fold at the protein level) and decreased (by ~53% at the mRNA and 50% at the protein level) in parallel with miR-23a overexpression and knockdown, respectively. In contrast, overexpression and knockdown of Twist had no significant effect on the expression of miR-23a (Fig. 1). Our pilot study suggested that miR-23a would regulate Twist expression in TSCC cells by a JNK-dependent mechanism (data not shown). Therefore, we included a selective JNK inhibitor SP600125 (10 μM) in all experiments in the present study (15). As shown in Fig. 1, the JNK inhibitor had no significant effect on the constitutive expression level of miR-23a. However, it abolished miR-23a-induced Twist expression in the TSCC cells (Figs. 1 and 2).
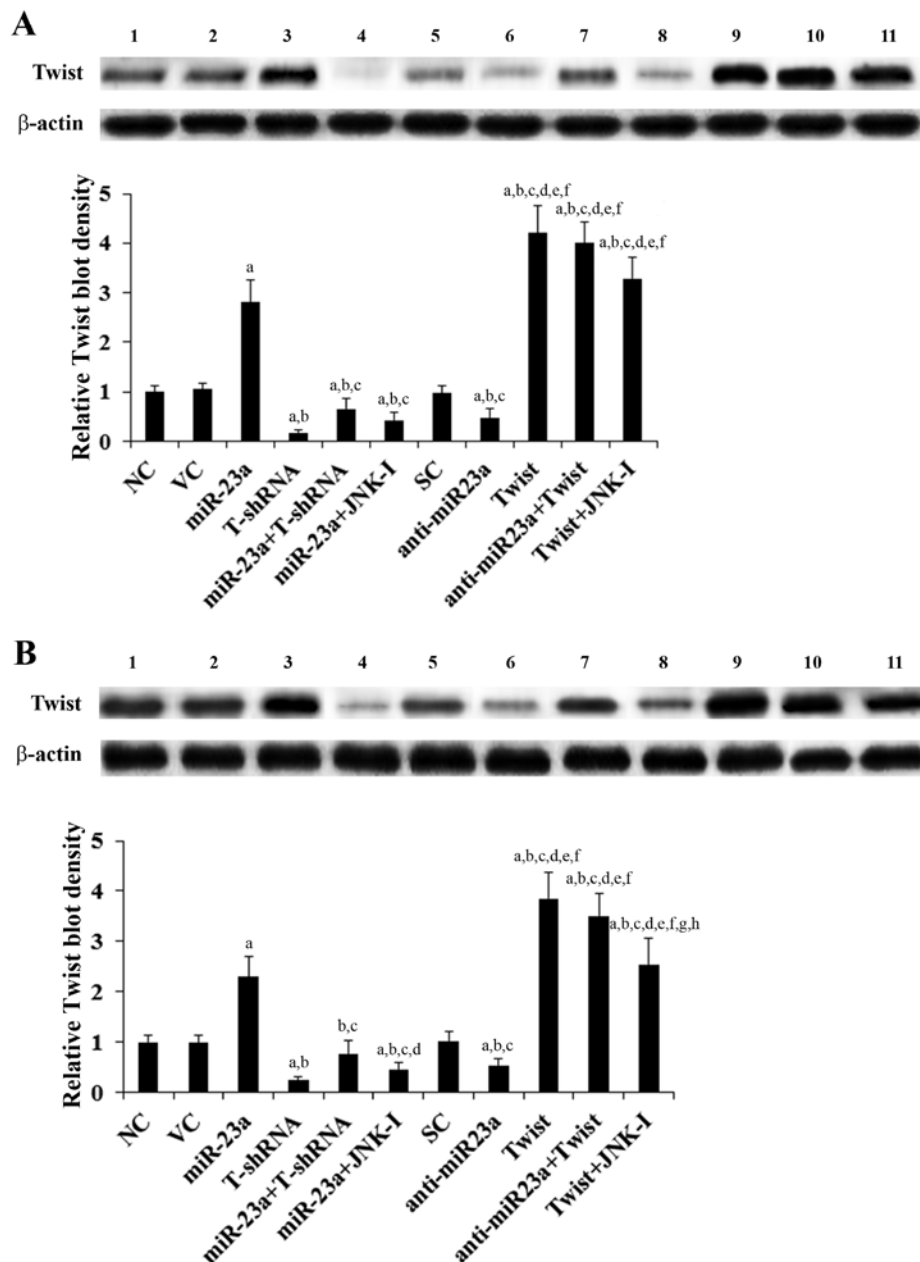
Figure 2. Protein levels of Twist in tongue squamous cell carcinoma (TSCC) cells with overexpression and knockdown of miR-23a and/or Twist. In (A) SCC-4 and (B) Tca8113 human TSCC cells, the protein levels of Twist were determined with western blot analysis in normal control cells (NC, lane 1), cells transduced with empty lentiviral vector pMIR and scramble control lentiviral shRNA (VC, lane 2), cells overexpressing miR-23a (transduced with lentiviral pMIRH23aPA-1) (lane 3), cells stably transduced with lentiviral Twist-shRNA (T-shRNA, lane 4), cells stably transduced with lentiviral Twist-shRNA and overexpressing miR-23a (miR-23a+T-shRNA, lane 5), cells overexpressing miR-23a and treated with selective c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10 μM) for 30 min (miR-23a+JNK-I, lane 6), cells transduced with lentiviral vector pMIRZIP expressing scramble control hairpin vector sequences and transfected with empty pcDNA3.1 vector (SC, lane 7), cells overexpressing lentiviral shRNA against miR-23a (transduced with lentiviral pMIRZIP-23a) (anti-miR23a, lane 8), cells overexpressing Twist (stably transfected with pcDNA3.1-Twist expression vector) (lane 9), cells overexpressing anti-miR23a and Twist (lane 10), and cells overexpressing Twist and treated with SP600125 (10 μM) for 30 min (Twist+JNK-I, lane 11). β-actin blotting was used as a loading control. Density of the Twist blot was normalized against that of the β-actin blot to obtain a relative blot density, which was expressed as a fold-change to that of NC (designated as 1). Three independent experiments were performed for each western blot analysis. Data values are expressed as means ± SD. [a]$p<0.05$ vs. controls (NC, VC and SC); [b]$p<0.05$ vs. miR-23a; [c]$p<0.05$ vs. T-shRNA; [d]$p<0.05$ vs. miR-23a+T-shRNA; [e]$p<0.05$ vs. miR-23a+JNK-I; [f]$p<0.05$ vs. anti-miR23a; [g]$p<0.05$ vs. Twist; [h]$p<0.05$ vs. anti-miR23a+Twist.

Effects of overexpression and knockdown of miR-23a and Twist on JNK activity in TSCC cells. The above results suggested that miR-23a regulates Twist expression in TSCC cells in a JNK-dependent manner. Therefore, we next examined the individual effect of and interaction between miR-23a and Twist on JNK activity, which was measured by phosphorylation of c-Jun, a substrate of JNK (13). As

evidenced by the increased level of phosphorylated c-Jun, overexpression of miR-23a respectively induced JNK activity by 2.4- and 2.1-fold in the SCC-4 and Tca8113 cells, which was abolished by SP600125 (10 μM) yet not knockdown of Twist (Fig. 3). On the other hand, knockdown of miR-23a decreased JNK activity by ~70% in both SCC-4 and Tca8113 cells, which was not significantly affected by overexpression
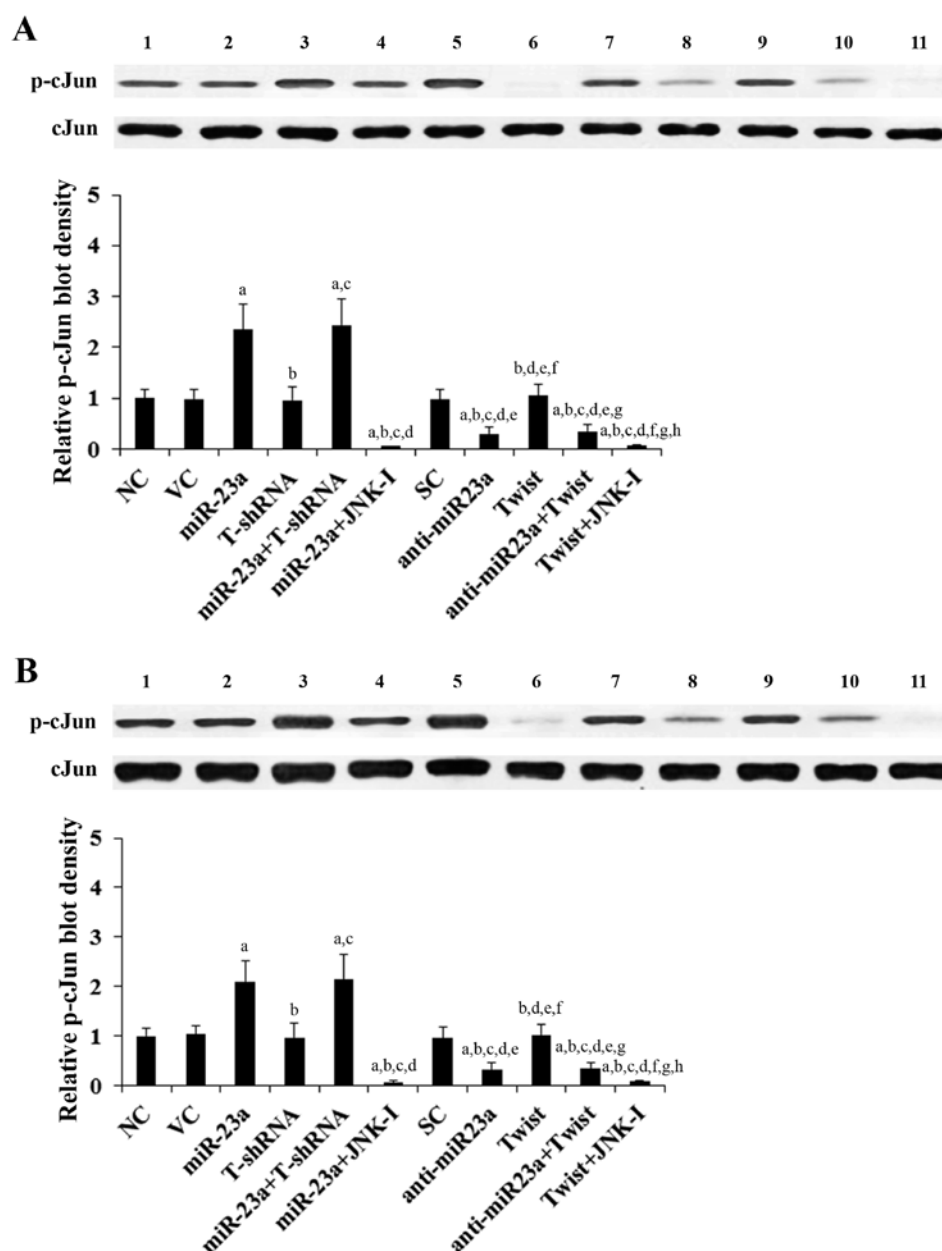
Figure 3. Effect of overexpression and knockdown of miR-23a and/or Twist on c-Jun N-terminal kinase (JNK) activity in tongue squamous cell carcinoma (TSCC) cells. In (A) SCC-4 and (B) Tca8113 TSCC cells, the JNK activity was determined by measuring phosphorylation of c-Jun, a substrate of JNK. The levels of phosphorylated c-Jun (p-cJun2) were determined with western blot analysis in normal control cells (NC, lane 1), cells transduced with empty lentiviral vector pMIR and scramble control lentiviral shRNA (VC, lane 2), cells overexpressing miR-23a (transduced with lentiviral pMIRH23aPA-1) (lane 3), cells stably transduced with lentiviral Twist-shRNA (T-shRNA, lane 4), cells stably transduced with lentiviral Twist-shRNA and overexpressing miR-23a (miR-23a+T-shRNA, lane 5), cells overexpressing miR-23a and treated with selective c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10 μM) for 30 min (miR-23a+JNK-I, lane 6), cells transduced with lentiviral vector pMIRZIP expressing scramble control hairpin vector sequences and transfected with empty pcDNA3.1 vector (SC, lane 7), cells overexpressing lentiviral shRNA against miR-23a (transduced with lentiviral pMIRZIP-23a) (anti-miR23a, lane 8), cells overexpressing Twist (stably transfected with pcDNA3.1-Twist expression vector) (lane 9), cells overexpressing anti-miR23a and Twist (lane 10), and cells overexpressing Twist and treated with SP600125 (10 μM) for 30 min (Twist+JNK-I, lane 11). Density of the p-cJun blot was normalized against that of c-Jun (cJun) to obtain a relative blot density, which was expressed as a fold-change to that of NC (designated as 1) as a measure of the JNK activity. Each experiment was repeated three times in duplicates. Data values are expressed as means ± SD. [a]p<0.05 vs. controls (NC, VC and SC); [b]p<0.05 vs. miR-23a; [c]p<0.05 vs. T-shRNA; [d]p<0.05 vs. miR-23a+T-shRNA; [e]p<0.05 vs. miR-23a+JNK-I; [f]p<0.05 vs. anti-miR23a; [g]p<0.05 vs. Twist; [h]p<0.05 vs. anti-miR23a+Twist.

of Twist (Fig. 3). Compared with the controls, overexpression and knockdown of Twist showed no significant effect on JNK activity (Fig. 3).

Effects of overexpression and knockdown of miR-23a and Twist on TSCC cell chemoresistance to cisplatin. To explore the individual effect of and interaction between miR-23a and

Twist on TSCC chemoresistance, we examined cisplatin IC$_{50}$ in TSCC cells. A higher IC$_{50}$ value was considered to correspond to clinical chemoresistance to cisplatin (16). As shown in Fig. 4, following 48 h of cisplatin treatment, the cisplatin IC$_{50}$ values for SCC-4 and Tca8113 cells were 6.2 and 7.1 μM, respectively. Overexpression of miR-23a significantly increased the IC$_{50}$ to 17.5 and 16.0 μM, respectively, which was abolished by
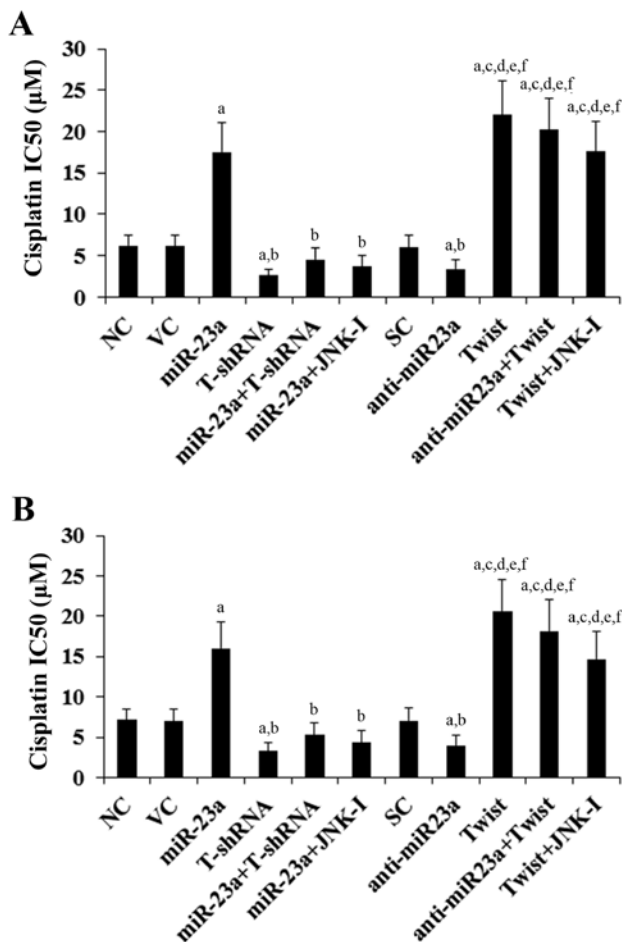
Figure 4. Effect of overexpression and knockdown of miR-23a and/or Twist on cisplatin chemoresistance in tongue squamous cell carcinoma (TSCC) cells. (A) SCC-4 and (B) Tca8113 TSCC cells were treated with or without various concentrations of cisplatin for 48 h. The half maximal inhibitory concentration (IC_{50}) values were determined in normal control cells (NC), cells transduced with empty lentiviral vector pMIR and scramble control lentiviral shRNA (VC), cells overexpressing miR-23a (transduced with lentiviral pMIRH23aPA-1), cells stably transduced with lentiviral Twist-shRNA (T-shRNA), cells stably transduced with lentiviral Twist-shRNA and overexpressing miR-23a (miR-23a+T-shRNA), cells overexpressing miR-23a and treated with selective c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10 μM) for 30 min (miR-23a+JNK-I), cells transduced with lentiviral vector pMIRZIP expressing scramble control hairpin vector sequences and transfected with empty pcDNA3.1 vector (SC), cells overexpressing lentiviral shRNA against miR-23a (anti-miR23a; transduced with lentiviral pMIRZIP-23a), cells overexpressing Twist (stably transfected with pcDNA3.1-Twist expression vector), cells overexpressing anti-miR23a and Twist, and cells overexpressing Twist and treated with SP600125 (10 μM) for 30 min (Twist+JNK-I). Each experiment was repeated three times in duplicates. Data values are expressed as means ± SD. [a]$p<0.05$ vs. controls (NC, VC and SC); [b]$p<0.05$ vs. miR-23a; [c]$p<0.05$ vs. T-shRNA; [d]$p<0.05$ vs. miR-23a+T-shRNA; [e]$p<0.05$ vs. miR-23a+JNK-I; [f]$p<0.05$ vs. anti-miR23a.

knockdown of Twist or SP600125 (10 μM) (Fig. 4). In contrast, knockdown of miR-23a respectively decreased the IC_{50} to 3.3 and 4.0 μM, which was completely reversed by overexpression of Twist (Fig. 4). Overexpression of Twist respectively increased the cisplatin IC_{50} to 22.1 and 20.6 μM in SCC-4 and Tca8113 cells, while knockdown of Twist decreased the IC_{50} to 2.6 and 3.3 μM, respectively (Fig. 4).

Effects of overexpression and knockdown of miR-23a and Twist on cisplatin-induced apoptosis in TSCC cells. We next

examined the individual effect of and interaction between miR-23a and Twist on cisplatin-induced apoptosis in the TSCC cells. Under normal culture conditions, overexpression and knockdown of miR-23a and Twist showed no significant effect on TSCC cell apoptosis compared with the controls (data not shown). After 30 h of cisplatin (20 μM) treatment, the percentages of apoptotic cells in SCC-4 (Fig. 5) and Tca8113 (Fig. 6) cells were 20.1 and 22.7%, respectively. Overexpression of miR-23a significantly decreased cell apoptosis to 8.3 and 11.9%, respectively, which was completely reversed by knockdown of Twist or by SP600125 (10 μM) (Figs. 5 and 6). In contrast, knockdown of miR-23a respectively increased cell apoptosis to 31.2 and 34.0%, which was abolished by overexpression of Twist (Figs. 5 and 6). Overexpression of Twist decreased apoptosis of SCC-4 (Fig. 5) and Tca8113 (Fig. 6) cells to 6.7 and 8.1%, respectively, while knockdown of Twist increased cell apoptosis to 41.4 and 39.6%, respectively.

Discussion

TSCC is associated with poorer survival and a lower rate of local tumor control than other sites of head and neck cancer (1). Innate or acquired resistance to cisplatin, a standard chemotherapy agent for TSCC, is common in patients with TSCC (4-6). Increased expression of miR-23a reportedly promotes cisplatin chemoresistance in TSCC cells (3). High expression of Twist is also associated with cancer chemoresistance (10) and poor prognosis of TSCC patients (11). In the present study, we demonstrate that miR-23a promotes cisplatin chemoresistance in TSCC cells largely through Twist.

We employed cisplatin IC_{50} as a measure of cisplatin chemoresistance in TSCC cells. A higher IC_{50} value was considered to correspond to clinical chemoresistance to cisplatin (16). As evidenced by overexpression and knockdown experiments, miR-23a and Twist individually increased cisplatin IC_{50}/cisplatin chemoresistance in TSCC cells. Knockdown of Twist abolished the chemoresistance-promoting effect of overexpressing miR-23a, while overexpression of Twist completely reversed the inhibitory effect of knocking down miR-23a on cisplatin chemoresistance. The findings indicate that Twist is functionally downstream of miR-23a and largely mediates the promoting effects of miR-23a on cisplatin chemoresistance in TSCC cells, which corroborates our finding that miR-23a induces Twist expression in TSCC cells. In addition, miR-23a and Twist individually showed a protective effect against cisplatin-induced apoptosis in TSCC cells. Knockdown of Twist abolished the protective effect of overexpressing miR-23a, while overexpression of Twist completely reversed the apoptosis-enhancing effect of knocking down miR-23a. The protective effect of miR-23a/Twist signaling against cisplatin-induced apoptosis serves as a mechanistic explanation for miR-23a/Twist-induced cisplatin chemoresistance in TSCC cells.

Overexpression and knockdown of miR-23a in TSCC cells respectively increased and decreased the expression of Twist at both the mRNA and the protein levels, yet not vice versa. In addition, a selective JNK inhibitor readily abolished miR-23a-induced Twist expression in TSCC cells without significantly altering the expression of miR-23a, indicating that miR-23a induces Twist expression in a JNK-dependent
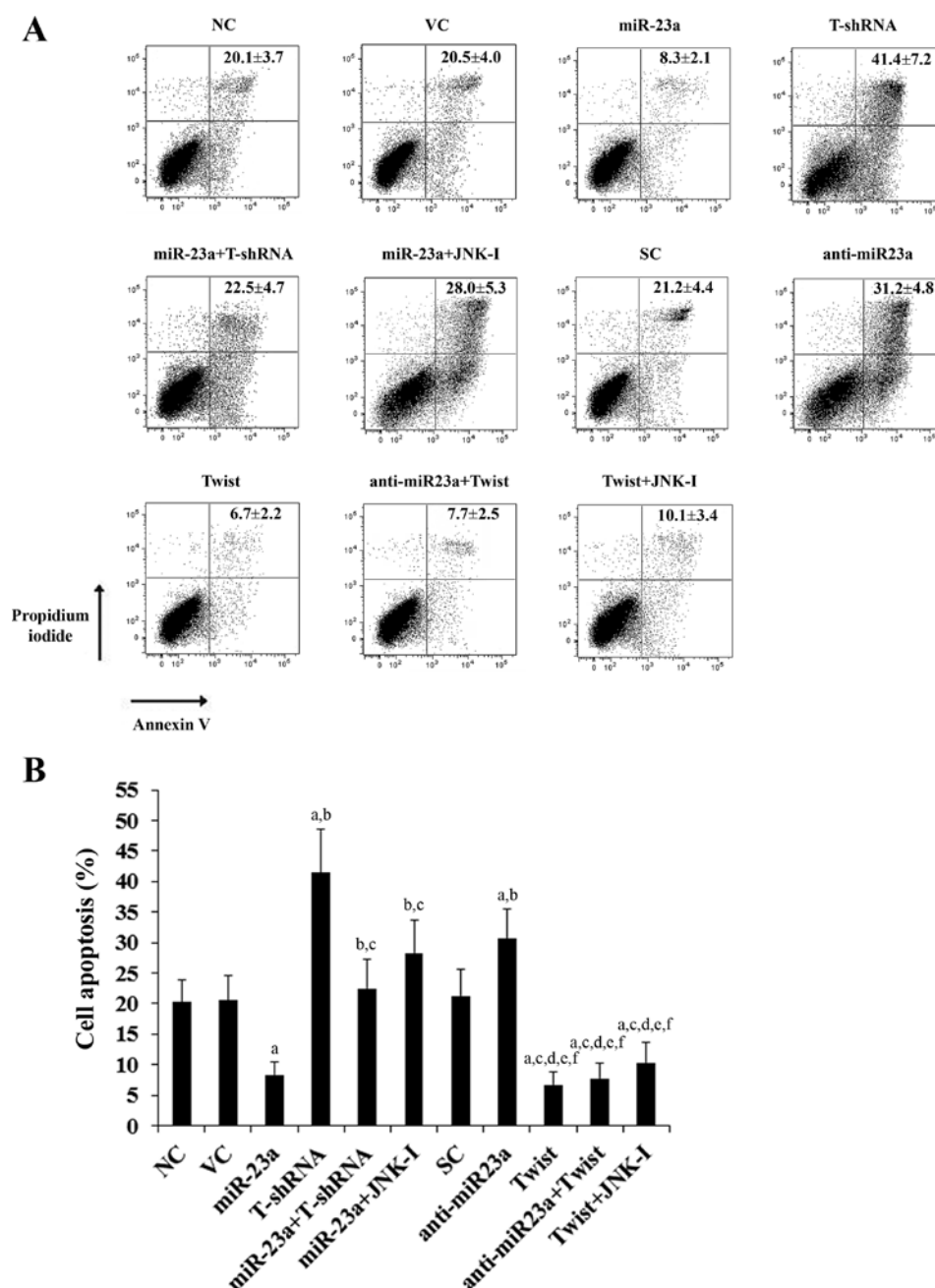
Figure 5. Effect of overexpression and knockdown of miR-23a and/or Twist on apoptosis of SCC-4 tongue squamous cell carcinoma (TSCC) cells. SCC-4 TSCC cells were treated with 20 μM of cisplatin for 30 h. (A) Cell apoptosis was quantitated using Annexin V/propidium iodide staining coupled with flow cytometric analysis in normal control cells (NC), cells transduced with empty lentiviral vector pMIR and scramble control lentiviral shRNA (VC), cells overexpressing miR-23a (transduced with lentiviral pMIRH23aPA-1), cells stably transduced with lentiviral Twist-shRNA (T-shRNA), cells stably transduced with lentiviral Twist-shRNA and overexpressing miR-23a (miR-23a+T-shRNA), cells overexpressing miR-23a and treated with selective c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10 μM) for 30 min (miR-23a+JNK-I), cells transduced with lentiviral vector pMIRZIP expressing scramble control hairpin vector sequences and transfected with empty pcDNA3.1 vector (SC), cells overexpressing lentiviral shRNA against miR-23a (anti-miR23a; transduced with lentiviral pMIRZIP-23a), cells overexpressing Twist (stably transfected with pcDNA3.1-Twist expression vector), cells overexpressing anti-miR23a and Twist, and cells overexpressing Twist and treated with SP600125 (10 μM) for 30 min (Twist+JNK-I). Each experiment was repeated three times in duplicates. Sums of percentages of early apoptotic cells (left lower quadrant) and late apoptotic cells/dead cells (right upper quadrant) are expressed as means ± SD in the right upper quadrant of each dot figure. (B) Sums of the percentages of early apoptotic cells (left lower quadrant) and late apoptotic cells/dead cells (right upper quadrant) are shown as percentages of cell apoptosis in the histograms (means ± SD). [a]p<0.05 vs. controls (NC, VC and SC); [b]p<0.05 vs. miR-23a; [c]p<0.05 vs. T-shRNA; [d]p<0.05 vs. miR-23a+T-shRNA; [e]p<0.05 vs. miR-23a+JNK-I; [f]p<0.05 vs. anti-miR23a.

manner in TSCC cells. How miR-23a induces the expression of Twist through JNK in TSCC cells will be explored by us in subsequent studies.

While selective JNK inhibitor SP600125 cancelled the promoting effects of miR-23a on cisplatin chemoresistance in TSCC cells, overexpression of Twist still significantly augmented cisplatin chemoresistance in the presence of SP600125. The results suggest that miR-23a and Twist act functionally upstream and downstream of JNK, respectively. This is in agreement with our findings that while overexpression and knockdown of miR-23a respectively increased and decreased JNK activity, Twist showed no significant effect
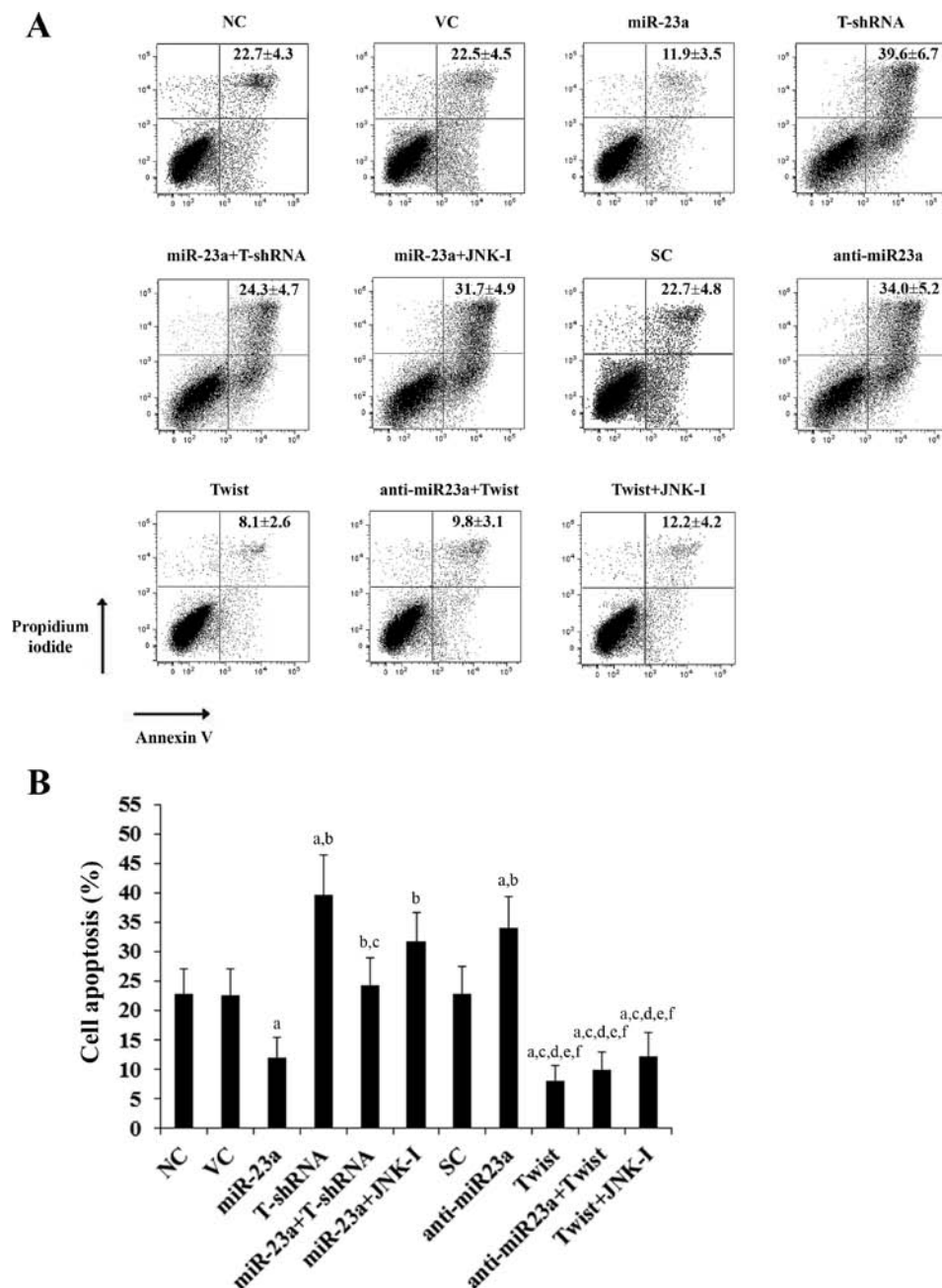
Figure 6. Effect of overexpression and knockdown of miR-23a and/or Twist on apoptosis of Tca8113 tongue squamous cell carcinoma (TSCC) cells. Tca8113 TSCC cells were treated with 20 µM of cisplatin for 30 h. (A) Cell apoptosis was quantitated using Annexin V/propidium iodide staining coupled with flow cytometric analysis in normal control cells (NC), cells transduced with empty lentiviral vector pMIR and scramble control lentiviral shRNA (VC), cells overexpressing miR-23a (transduced with lentiviral pMIRH23aPA-1), cells stably transduced with lentiviral Twist-shRNA (T-shRNA), cells stably transduced with lentiviral Twist-shRNA and overexpressing miR-23a (miR-23a+T-shRNA), cells overexpressing miR-23a and treated with selective c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10 µM) for 30 min (miR-23a+JNK-I), cells transduced with lentiviral vector pMIRZIP expressing scramble control hairpin vector sequences and transfected with empty pcDNA3.1 vector (SC), cells overexpressing lentiviral shRNA against miR-23a (anti-miR23a; transduced with lentiviral pMIRZIP-23a), cells overexpressing Twist (stably transfected with pcDNA3.1-Twist expression vector), cells overexpressing anti-miR23a and Twist, and cells overexpressing Twist and treated with SP600125 (10 µM) for 30 min (Twist+JNK-I). Each experiment was repeated three times in duplicates. Sums of the percentages of early apoptotic cells (left lower quadrant) and late apoptotic cells/dead cells (right upper quadrant) are expressed as means ± SD in the right upper quadrant of each dot figure. (B) Sums of the percentages of early apoptotic cells (left lower quadrant) and late apoptotic cells/dead cells (right upper quadrant) are shown as percentages of cell apoptosis in the histogram (means ± SD). [a]$p<0.05$ vs. controls (NC, VC and SC); [b]$p<0.05$ vs. miR-23a; [c]$p<0.05$ vs. T-shRNA; [d]$p<0.05$ vs. miR-23a+T-shRNA; [e]$p<0.05$ vs. miR-23a+JNK-I; [f]$p<0.05$ vs. anti-miR23a.

on JNK activity in TSCC cells. This is also in agreement with a previous report that JNK signaling may control the expression of twist (17). Previous studies have suggested that the JNK signaling pathway is clearly a basis for resistance to DNA-damaging drugs (18). Our findings indicate that JNK mediates miR-23a-induced Twist expression in TSCC

cells, which markedly promotes cisplatin chemoresistance. Therefore, the functional role of JNK signaling in cisplatin chemoresistance in TSCC cells is at least partially fulfilled through miR-23a/Twist signaling.

Yu *et al* demonstrated that miR-23a functions as an upstream regulator of DNA topoisomerase IIβ to induce cisplatin chemo-

resistance in TSCC cells (3). In the present study, we showed that miR-23a promotes cisplatin chemoresistance in TSCC cells through Twist. Thus, whether and how TOP2B and Twist would interact to impact cisplatin chemoresistance in TSCC cells would be a significant topic for future studies. Cisplatin elicits DNA repair mechanisms by crosslinking DNA, which in turn activates apoptosis when repair proves impossible (19). In the present study, we only examined the effect of miR-23a/ Twist signaling on cisplatin chemoresistance in TSCC cells. It is unclear whether miR-23a/Twist would impact chemoresistance to other types of chemotherapy agents. Further studies with more types of chemotherapy agents and TSCC cell lines would elucidate this issue. In addition, since Twist has been associated with chemoresistance in a variety of cancers such as nasopharyngeal, breast, prostate and lung cancer (10,20), it is worth defining the role of miR-23a/Twist signaling in other cancer types besides TSCC in future studies.

In conclusion, the present study for the first time demonstrated that miR-23a promotes cisplatin chemoresistance and protects against cisplatin-induced apoptosis in TSCC cells through inducing Twist expression by a JNK-dependent mechanism. It adds new insights into the molecular mechanisms underlying TSCC chemoresistance.

Acknowledgements

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