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TIMP-3 Increases the Chemosensitivity of Laryngeal Carcinoma to Cisplatin via Facilitating Mitochondria-Dependent Apoptosis

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Laryngeal carcinoma is a type of head and neck carcinoma with a high incidence and mortality. Chemotherapy treatments of human laryngeal carcinoma may fail due to the development of chemoresistance. Tissue inhibitor of metalloproteinase 3 (TIMP-3) has been shown to be implicated in a number of pathological processes typical for cancer. The present study aims to investigate the involvement of TIMP-3 in the chemoresistance of laryngeal carcinoma. We showed that TIMP-3 expression was significantly decreased in chemoresistant laryngeal carcinoma tissues compared with chemosensitivity tissues. Patients with low TIMP-3 expression exhibited poorer overall survival than those with high TIMP-3 expression. Moreover, cisplatin-resistant Hep-2 cells (Hep-2/R) were associated with the inhibition of mitochondrial membrane potential (MtMP) depolarization after cisplatin challenge. In addition, cisplatin resulted in a more pronounced mitochondrial cytochrome c release into the cytoplasm in Hep-2 cells than in their resistant variants. Overexpression of TIMP-3 by an adenovirus encoding TIMP-3 cDNA remarkably enhanced cisplatin-induced apoptosis, cytochrome c release, and caspase activation in Hep-2/R cells, thereby sensitizing cancer cells to cisplatin. On the other hand, downregulation of TIMP-3 markedly inhibited cisplatin-induced apoptosis in Hep-2 cells through attenuating mitochondria-dependent pathway activation. Taken together, these results demonstrate that decreased TIMP-3 expression may contribute to cisplatin resistance via inhibition of mitochondria-dependent apoptosis, indicating that forced TIMP-3 expression may be a useful strategy to improve the efficacy of cisplatin to treat laryngeal carcinoma.

Key words: Cisplatin; Chemoresistance; Laryngeal carcinoma; Mitochondria; Apoptosis; Tissue inhibitor of metalloproteinase 3 (TIMP-3)

INTRODUCTION

Laryngeal carcinoma takes the first place among the head and neck cancers and accounts for approximately 1–2.5% of all malignancies throughout the body[1,2]. For patients in the early stage of laryngeal carcinoma, surgery is suggested to be the most effective treatment approach[3]. Unfortunately, most patients are diagnosed with advanced stage laryngeal carcinoma at the first diagnosis, and the therapeutic effect of surgery at this stage is poor due to the wide metastasis of cancer cells[1,4]. Currently, combined therapies, such as chemotherapy, radiotherapy, and possible adjuvant therapy, are usually used in the advanced stage[5]. However, the overall survival rate has not improved because of the development of resistance to chemotherapy[3,4,6]. Thus, a better understanding of the molecular mechanisms of chemoresistance is extremely important to improve the therapeutic efficacy of treatments for laryngeal carcinoma.

Accumulating evidence has indicated that matrix metalloproteinases (MMPs) are critical for metastasis in several cancers, including laryngeal carcinoma, and upregulation of MMPs is associated with poor prognosis[7,8]. The tissue inhibitor of metalloproteinases (TIMPs) inhibits MMP activity and subsequently deregulates extracellular matrix formation[9]. Among the four subtypes of TIMPs, TIMP-3 not only suppresses MMPs but also inhibits a disintegrin and metalloproteinase (ADAM) activity, which is not regulated by the other TIMP members[8,10]. Previous studies have demonstrated that TIMP-3 influences the progression of cancers, such as tumor growth, angiogenesis, and invasion[11]. Abnormal expression or deregulation of TIMP-3 can be observed in several kinds

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of cancers[11,12]. However, no studies have yet investigated the correlation between TIMP-3 and the chemoresistance of laryngeal carcinoma. In this study, we aim to explore the underlying mechanisms of chemoresistance of laryngeal carcinoma and to examine whether TIMP-3 involves the cisplatin resistance in laryngeal carcinoma cells.

MATERIALS AND METHODS

Laryngeal Carcinoma Tissues

The research protocols were approved by the Ethics Committee of Nanjing University (Nanjing, P.R. China) and performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Informed consent was obtained from each patient. Human laryngeal carcinoma tissues were collected from patients during clinical surgery between 2010 and 2012 in the Department of Otolaryngology Head and Neck. Sixty-five patients were divided into two groups based on the follow-up visits from 2010: (1) patients with a good response to chemotherapy and a survival time of more than 2 years ($n=27$); (2) patients with a poor response to chemotherapy, who died within 3 years due to chemoresistance ($n=38$). The surgical specimens were fixed with 4% paraformaldehyde for immunohistochemical examination or stored in liquid nitrogen for quantitative real-time PCR (qPCR) and real-time PCR analysis.

Immunohistochemistry

The frozen slides (5 μm) of laryngeal carcinoma tissues were rehydrated in phosphate-buffered saline (PBS) and incubated with 0.3% hydrogen peroxide aqueous solution to block endogenous peroxidase activity. The slides were added with 2% nonimmune serum solution (Zhongshan Jinqiao Bio-Technology Co. Ltd., Beijing, P.R. China) at room temperature to block nonspecific binding, and then were incubated with TIMP-3 antibody overnight at 4°C. After three washes with PBS, the slides were incubated with biotinylated secondary antibody and visualized with the streptavidin–peroxidase reaction using 3,3′-diaminobenzidine (all from Zhongshan Jinqiao Bio-Technology Co. Ltd.). Negative controls included incubation with a nonspecific immunoglobulin of the same isotype as the primary antibody. The slides were observed and examined under a light microscope (magnification: 400×) (IX70; Olympus, Tokyo, Japan).

Quantitative Real-Time PCR

Total RNA from the human laryngeal carcinoma tissues was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The isolated RNA was determined by UV spectrometry, and 2 ug of RNA was reverse transcribed to cDNA using the ReverTra ACE qPCR RT Kit (Toyobo, Osaka, Japan). qPCR was performed with the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Fast SYBR® Green Master Mix Kit (Applied Biosystems). Amplifications were carried out with the following cycling conditions: 95°C for 10 min followed by 36 amplification cycles of denaturation (95°C for 10 s), annealing (60°C for 1 min), and extension (62°C for 30 s). The TIMP-3 mRNA expression was normalized to the mRNA levels of GAPDH, and the data were calculated using the $2-\Delta\Delta CT$ method. The specific primer sequences used for the amplification were as follows: TIMP-3, 5′-GTGGTCAGCCTCTCTCACAC-3′ and 5′-CCAAGGCCTAACAGATGGGG-3′; GAPDH, 5′-GGGCACGAAGGCTCATCATT-3′ and 5′-AGAAGGCTGGGGCTCATTTG-3′.

Cell Culture

Human laryngeal cell carcinoma line human epithelial type 2 (Hep-2) was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Grand Island, NY, USA) at 37°C in a 5% CO_2 incubator. The cisplatin-resistant Hep-2 (Hep-2/R) cells were developed from the parental Hep-2 and cultured in DMEM supplemented with 2 μM cisplatin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C with 5% CO_2. To exclude the inference of residual cisplatin, Hep-2/R cells were cultured in cisplatin-free medium for 2 weeks before experiments.

Western Blotting

Human laryngeal carcinoma tissues or cells were washed with ice-cold PBS and lysed with RIPA lysis buffer (Beyotime, Jiangsu, P.R. China) containing protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN, USA) at 4°C. Mitochondrial fractions were extracted using the Mitochondria Isolation Kit (Sigma-Aldrich) according to the supplier's instructions. The protein concentration was determined by Enhanced BCA Protein Assay Kit (Beyotime). An equal amount of protein was separated by 10%–12% SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Five percent nonfat milk was added to membranes to block nonspecific binding at room temperature for 1 h. The membranes were then incubated with the following primary antibodies: TIMP-3 (1:500), Cox-IV, GAPDH, β-actin (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA); cytochrome c, caspase 9, and caspase 3 (1:1,000; Cell Signaling Technology, Billerica, MA, USA). The immunoreactive proteins were detected with

horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit or rabbit anti-mouse; 1:1,000; Beyotime) and an enhanced chemiluminescence reagent (Pierce Biotech, Rockford, IL, USA). Densitometry of bands was quantified using ImageJ software (NIH, Bethesda, MD, USA).

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential (MtMP) was measured by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide (JC-1; Sigma-Aldrich) fluorescence staining, an indicator of the occurrence of apoptosis. JC-1 exhibits potential-dependent accumulation in mitochondria in viable cells, indicated by red fluorescence. In apoptotic cells, the dye outflows into the cytoplasm as a monomer and emits green fluorescence, signifying the breakdown of the MtMP. Thus, the red/green fluorescence intensity ratio indicates the change in MtMP. The MtMP measurement was performed using a confocal microscope or flow cytometry. Hep-2 cells or Hep-2/R cells were incubated with JC-1 working solution in the dark at 37°C for 15 min. After washing twice with PBS, the fluorescence was measured using a FV1000 confocal microscope (Olympus). For flow cytometry, cells were collected, washed with PBS, and resuspended in medium containing JC-1. After incubation at 37°C with 5% CO_2, cells were analyzed by flow cytometry (FACSCaliber; Becton Dickinson, Heidelberg, Germany).

Adenoviral Infection

Human TIMP-3 adenovirus harboring TIMP-3 cDNA was designed and purchased from Sunbio Medical Biotechnology (Shanghai, P.R. China). A negative control (Lacz) was obtained from Clontech (Palo Alto, CA, USA). To overexpress TIMP-3 in Hep-2/R cells, the cells were infected with TIMP-3 adenovirus or Lacz in FBS-free DMEM for 6 h and then transferred into complete medium and cultured for 48 h.

TIMP-3 siRNA Transfection

The stealth siRNA targeting human TIMP-3 (5′-CUA CTTCCCATATTCATTCATU-3′) was synthesized and purchased from Invitrogen. Hep-2 cells were transfected by electroporation with either TIMP-3 siRNA or negative siRNA using Nucleofector Kit (Lonza, Basel, Switzerland). The cells were then replaced with DMEM containing 10% FBS and incubated for 48 h at 37°C before cisplatin treatment.

Cell Viability

The viability of Hep-2 cells or Hep-2/R cells was measured by cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. After the indicated treatments, the cells were incubated with 10 μl of CCK-8 reagent for 4 h, at 37°C, 5% CO_2. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

Apoptosis Detection Assay

Cell apoptosis was evaluated using the FITC-Annexin-V and propidium iodide (PI) Apoptosis Detection Kit (Beyotime) by FACSCaliber flow cytometry (Becton Dickinson) according to the manufacturer's instruction. Hep-2 cells or Hep-2/R cells were harvested, washed three times with PBS, and incubated with annexin V and PI in the dark for 20 min at room temperature. Apoptotic cells were counted with flow cytometry, and the data were analyzed by the accompanied software (Becton Dickinson).

Statistical Analysis

Data were expressed as mean value ± standard error of mean (SEM) and compared by two-tailed Student's *t*-test or one-way ANOVA, followed by the Bonferroni multiple comparison test. Statistical analysis was performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Survival curves were estimated using Kaplan–Meier method and compared by log rank test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Decreased Expression of TIMP-3 in Chemoresistant Laryngeal Carcinoma Tissues

TIMP-3 has been suggested to play a critical role in cancerogenesis[11,12]. To unveil the role of TIMP-3 in the chemoresistance of laryngeal carcinoma, the expression of TIMP-3 in chemosensitive and chemoresistant laryngeal carcinoma tissues was determined. Immunohistochemistry showed that TIMP-3 expression was significantly decreased in chemoresistant laryngeal carcinoma tissues compared with chemosensitive tissues (Fig. 1A). Similarly, qPCR also revealed lower mRNA expression of TIMP-3 in chemoresistant tissues than in chemosensitive tissues (Fig. 1B). The above results were further confirmed by Western blotting analysis, revealing that the protein expression of TIMP-3 was markedly reduced in chemoresistant laryngeal carcinoma tissues (Fig. 1C). Importantly, Kaplan–Meier survival analysis demonstrated that low TIMP-3 patients were significantly connected with poor prognosis, indicating that the laryngeal carcinoma patients with high TIMP-3 expression have obviously longer overall survival time than those with low TIMP-3 expression (Fig. 1D). These results suggest that decreased expression of TIMP-3 may be associated with chemoresistance of laryngeal carcinoma.

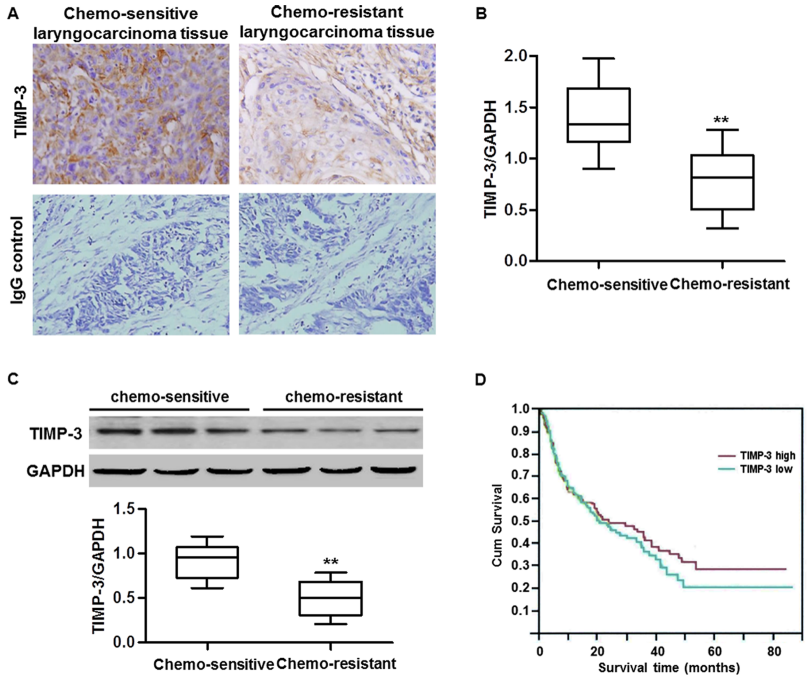

Figure 1. The expression of tissue inhibitor of metalloproteinase 3 (TIMP-3) was correlated with cisplatin resistance in laryngeal carcinoma tissues. (A) Immunohistochemical protein expression of TIMP-3 in chemosensitive ($n=8$) and chemoresistant ($n=11$) laryngeal carcinoma tissues. (B) Quantitative real-time PCR and (C) Western blotting were used to analyze the endogenous expression of TIMP-1 in chemosensitive tissues ($n=27$) compared with chemoresistant tissues ($n=38$). Quantitative results of TIMP-3 mRNA and protein expression are shown. $^{**}p<0.01$ versus chemosensitive tissue. (D) The survival state of each patient was recorded during follow-up, and the survival curves are shown.

Cisplatin Resistance Was Related to Inhibition of MtMP Depolarization and Cytochrome c Release

The mitochondrial apoptosis pathway plays an important role in regulating the survival or death of cancer cells[13]. To investigate whether mitochondria-dependent apoptosis is involved in cisplatin resistance in laryngeal carcinoma cells, the effects of cisplatin on mitochondrial membrane permeability in Hep-2 cells and cisplatin-resistant Hep-2/R cells were examined. Confocal microscopy by JC-1 staining showed that there was no significant difference in the ratio of red to green fluorescence between Hep-2 cells and Hep-2/R cells under basal levels. Cisplatin treatment resulted in a marked decline in red/green fluorescence ratio, indicating MtMP depolarization. Notably, cisplatin-induced MtMP depolarization was inhibited in Hep-2/R cells, although cisplatin also led to a slight decrease in red/green fluorescence ratio in these cells (Fig. 2A and B). Similarly, flow cytometric analysis revealed that cisplatin dramatically increased the numbers of cells with collapsed MtMP in Hep-2 cells, but to less of an extent in their counterpart Hep-2/R cells (Fig. 2C and D). Loss of MtMP can induce the release of mitochondrial cytochrome c into cytoplasm[14]. We then measured the level of cytochrome c in the mitochondria and cytoplasm, respectively. After cisplatin treatment, the translocation of cytochrome c from mitochondria to the cytoplasm was significantly increased in cisplatin-sensitive Hep-2 cells, and this increase was suppressed in Hep-2/R cells (Fig. 2E and F). These data indicate that cisplatin-induced MtMP depolarization and cytochrome c release are more pronounced in Hep-2 cells than in cisplatin-resistant cells.

TIMP-3 Overexpression Increased the Sensitivity of Hep-2/R Cells to Cisplatin

To examine the function of TIMP-3 in cisplatin resistance, we upregulated TIMP-3 expression in Hep-2/R cells by infection with TIMP-3 adenovirus and determined its sensitivity to cisplatin. Western blotting showed that TIMP-3 adenovirus infection significantly increased TIMP-3 expression, while no increase in Lacz group was observed (Fig. 3A). As displayed in Figure 3B, overexpression of TIMP-3 had no effect on Hep-2/R cell viability under basal levels. However, in cisplatin-treated Hep-2/R cells, TIMP-3 upregulation markedly decreased the viability of Hep-2/R cells compared with Lacz treatment (Fig. 3B). Moreover, the cisplatin-induced apoptosis in Hep-2/R cells was also increased after TIMP-3 adenovirus treatment (Fig. 3C and D). To further investigate whether TIMP-3 decreases cisplatin

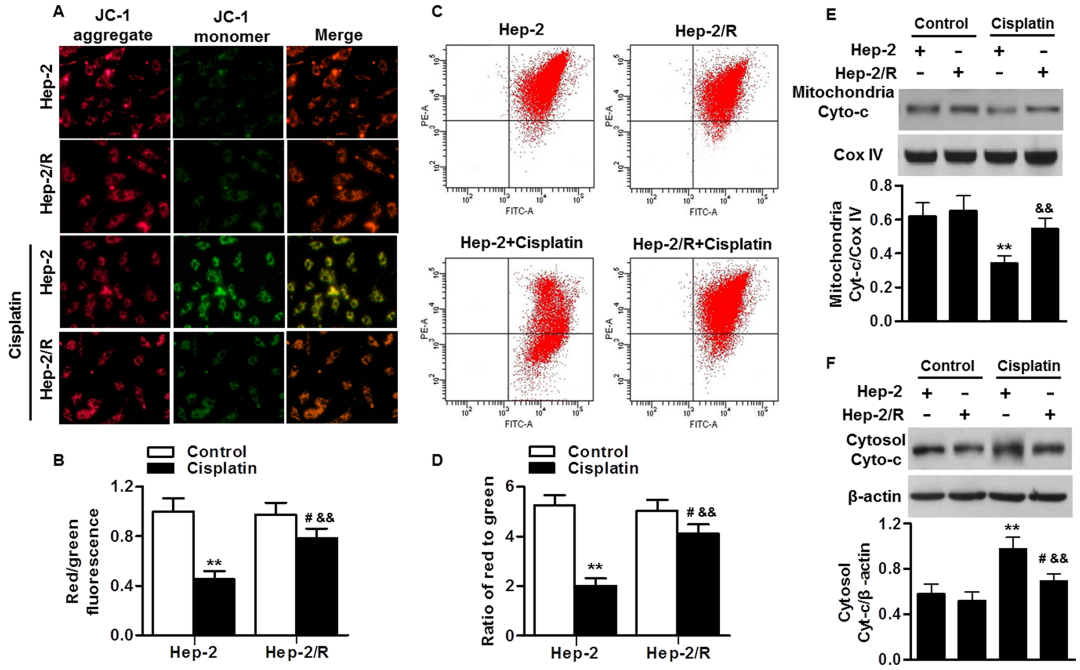

Figure 2. Cisplatin resistance was associated with inhibition of mitochondrial membrane potential (MtMP) depolarization and cytochrome c release. (A) Human epithelial type 2 (Hep-2) cells or cisplatin-resistant Hep-2/R cells were treated with or without cisplatin (2 μM) for 24 h. MtMP was measured by JC-1 staining. Confocal microscopy showed the representative images of JC-1 fluorescence. The aggregated JC-1 image is shown with red fluorescence, while the monomer image is shown by green fluorescence. (B) Quantitative analysis of the ratio of red to green fluorescence. (C) The MtMP measurement was performed using flow cytometry. (D) The bar diagram showed the ratio of JC-1 red fluorescence to green fluorescence. After the treatment mentioned in (A), the level of mitochondrial (E) and cytosol (F) cytochrome c was determined by Western blotting. $^{**}p<0.01$ versus Hep-2 cells, $\#p<0.05$ versus Hep-2/R cells, $\&\&p<0.01$ versus Hep-2 cells + cisplatin, $n=6$.

resistance in laryngeal carcinoma cells through regulating mitochondrial membrane permeability, cytochrome c release was determined. We found that cisplatin-induced mitochondrial cytochrome c release was markedly potentiated in TIMP-3 adenovirus-infected Hep-2/R cells (Fig. 3E and F). Release of mitochondrial cytochrome c into the cytoplasm is accompanied with activation of the caspase-dependent apoptosis pathway[14]. The results showed that cisplatin increased caspase 9 and caspase 3 cleavage. Although cleaved caspase 9 and caspase 3 remained unchanged after TIMP-3 adenovirus infection under basal levels, TIMP-3 overexpression obviously promoted caspase 9 and caspase 3 cleavage in the presence of cisplatin (Fig. 3G and H). These results suggest that TIMP-3 upregulation promotes the mitochondria-dependent apoptosis pathway activation and decreases cisplatin resistance in Hep-2/R cells.

Knockdown of TIMP-3 Attenuated Cisplatin-Induced Apoptosis in Hep-2 Cells

To further confirm the role of TIMP-3 in regulating cisplatin resistance in laryngeal carcinoma cells, cisplatin-sensitive Hep-2 cells were transfected with TIMP-3 siRNA before cisplatin challenge. TIMP-3 siRNA at 80 nM significantly decreased endogenous TIMP-3 expression more than 75% (Fig. 4A). Although knockdown of TIMP-3 had no effect on the viability of Hep-2 cells under basal levels, cisplatin-induced cell growth inhibition was markedly reversed after TIMP-3 siRNA treatment (Fig. 4B). Similarly, the annexin V/PI double staining by flow cytometry revealed that downregulation of TIMP-3 significantly inhibited cisplatin-induced apoptosis compared with negative siRNA group, with a decrease in the percentage of apoptotic cells from 38.2% to 18.1% (Fig. 4C and D). In addition, mitochondrial cytochrome c release induced by cisplatin was attenuated by TIMP-3 inhibition (Fig. 4E and F). As expected, an increased level of caspase 9 and caspase 3 cleavage in Hep-2 cells was also observed after cisplatin treatment, and this was dramatically inhibited after TIMP-3 downregulation (Fig. 4G and H). Overall, the data indicate that TIMP-3 knockdown decreases cisplatin sensitivity in laryngeal carcinoma cells.

DISCUSSION

Although treatment and diagnosis of laryngeal carcinoma have gradually improved, the incidence and mortality are still high, with a 5-year overall mortality rate

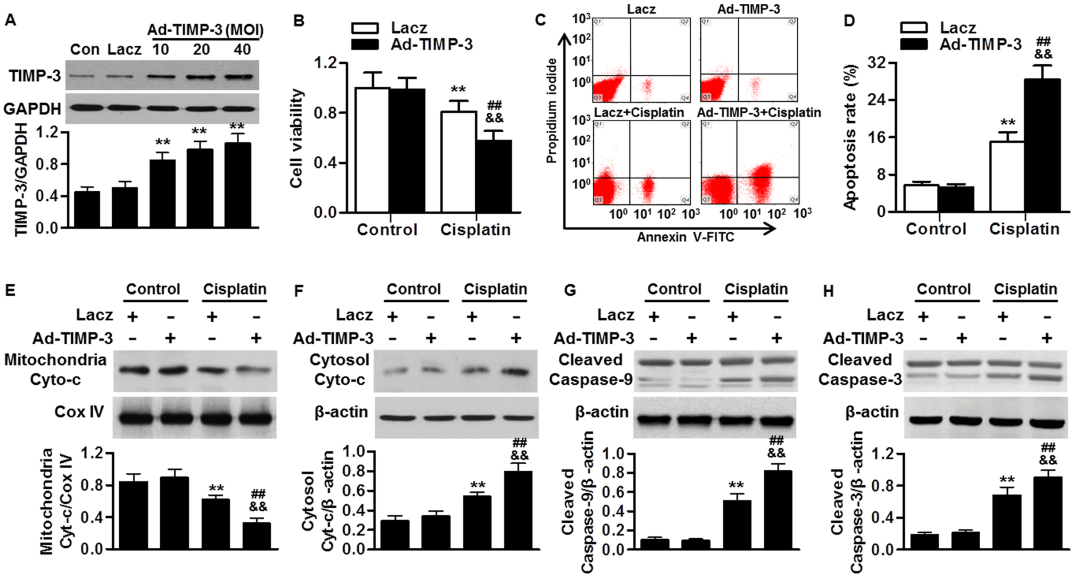

Figure 3. TIMP-3 upregulation increased the sensitivity of cisplatin-resistant laryngeal carcinoma cells to cisplatin treatment. (A) Cisplatin-resistant Hep-2/R cells were infected with different multiplicity of Infection (MOI; 10, 20, or 40) of TIMP-3 adenovirus (Ad-TIMP-3) for 48 h. Western blotting analysis of TIMP-3 expression. $^{**}p<0.01$ versus control, $n=6$. (B) The cells were infected with TIMP-3 adenovirus (20 MOI) for 48 h and then treated with cisplatin (2 μM) for 24 h. Cell viability was determined by cell counting kit-8 (CCK-8) assay. (C) Cell apoptosis was examined by annexin V/propidium iodide (PI) staining using flow cytometry. (D) Quantitative analysis of the percentage of apoptotic cells. (E) Mitochondrial and cytosol (F) cytochrome c release was analyzed by Western blotting. Western blotting analysis of the level of caspase 9 (G) and caspase 3 (H) cleavage. $^{**}p<0.01$ versus Lacz, $\#\#p<0.01$ versus Ad-TIMP-3, $\&\&p<0.01$ versus Lacz + cisplatin, $n=6$.

of ~40%[2]. Chemotherapy resistance and late metastasis in advanced stage are responsible for the poor prognosis of laryngeal carcinoma. In the present study, we investigated the effect of TIMP-3 on cisplatin resistance in laryngeal carcinoma cells. The results of this study are summarized as follows: (1) we demonstrate for the first time that cisplatin resistance in laryngeal carcinoma cells is associated with MtMP stabilization. (2) TIMP-3 expression is reduced in chemoresistant laryngeal carcinoma tissues compared with chemosensitivity tissues. (3) Upregulation of TIMP-3 promotes MtMP depolarization and mitochondrial cytochrome c release, which conferred cisplatin resistance in cisplatin-resistant laryngeal carcinoma cells. This study may unveil a key mechanism of the development of chemoresistance of laryngeal carcinoma.

Cisplatin, an inorganic molecule, is one of the most effective agents widely used for the treatment of various cancers, including laryngeal carcinoma[15]. It binds with nitrogen atoms of the DNA base, induces DNA damage, and thus triggers apoptosis in cancer cells[16]. Despite the achievement of satisfactory effects at the beginning of chemotherapy, cisplatin resistance still significantly limits its long-term application in the clinic[3,5]. Apoptosis acts as the most common form of programmed cell death, playing an important role in the regulation of cancer cell death[16,17]. Resistance to apoptosis is not only frequently observed in cancer cells but also thought to be the major phenotype of chemoresistance[13,18]. Mitochondria are an important sensor of intrinsic apoptosis[17,18]. Upon receiving a death signal, the mitochondria membrane permeability increases and allows mitochondrial cytochrome c to be released into the cytoplasm of the cells, leading to the triggering of the downstream pathways of apoptosis[19]. It has been documented that inhibition of mitochondria-dependent apoptosis contributes to the chemotherapy-resistant phenotype, particularly toward DNA-damaging agents[13,20]. To the best of our knowledge, the present study was the first to show that cisplatin-induced MtMP depolarization and subsequent cytochrome c release were more pronounced in cisplatin-sensitive cells than in their resistant counterparts. These results suggest that inhibition of mitochondria-dependent apoptosis may be a determinant of cisplatin resistance in laryngeal carcinoma.

Another significant finding of our study was that TIMP-3 expression could be reduced in chemoresistant laryngeal carcinoma tissues. Multiple studies have revealed that TIMP-3 level is associated with the progression of different cancers[10,12,21]. Recently, TIMP-3 mRNA expression was found to be reduced in chondrosarcoma tissues compared with normal individuals[10]. Importantly, in laryngeal squamous cell carcinoma, it was reported

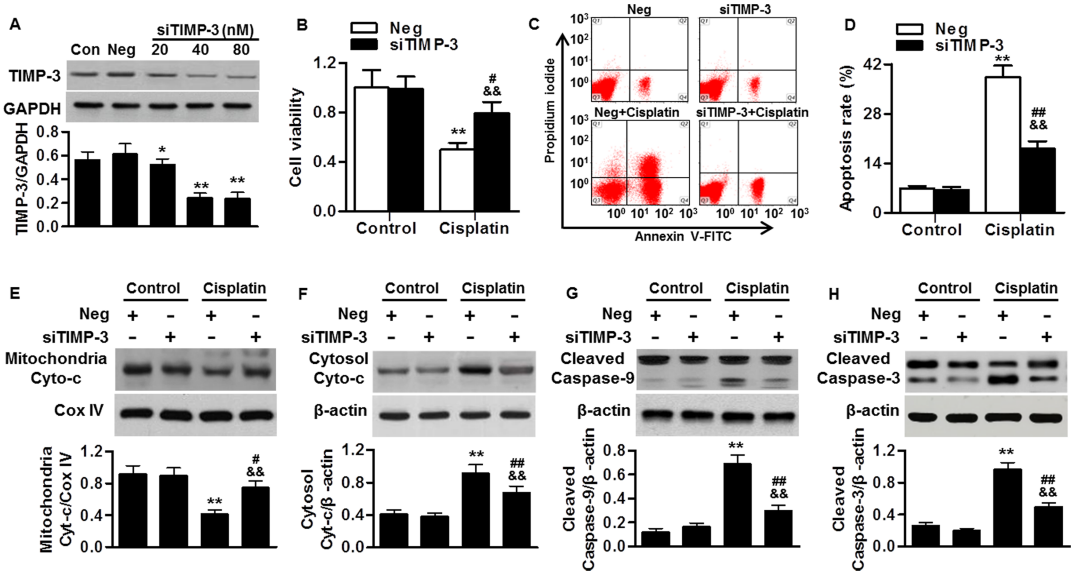

Figure 4. Knockdown of TIMP-3 decreased the sensitivity of laryngeal carcinoma cells to cisplatin treatment. (A) Cisplatin-sensitive Hep-2 cells were transfected with different concentrations (20, 40, or 80 nM) of TIMP-3 siRNA (siTIMP-3) or negative siRNA (Neg) for 48 h. The expression of TIMP-3 was determined by Western blotting. $*p<0.05$, $**p<0.01$ versus control, $n=5$. (B) Hep-2 cells were transfected with TIMP-3 siRNA (40 nM) for 48 h before cisplatin (2 μM) treatment for another 24 h. CCK-8 assay was used to detect the viability of Hep-2 cells. (C) Cell apoptosis was determined by annexin V/PI staining. (D) Quantitative analysis of the percentage of apoptotic cells. The level of cytochrome c in mitochondria (E) and cytoplasm (F) was examined by Western blotting. Western blotting analysis of the expression of cleaved caspase 9 (G) and cleaved caspase 3 (H). $**p<0.01$ versus Neg, $\#p<0.05$, $\#\#p<0.01$ versus siTIMP-3, $\&\&p<0.01$ versus Neg + cisplatin, $n=6$.

that high TIMP-3 expression was closely correlated with lymph node metastases[21]. Furthermore, Su et al. demonstrated that plasma TIMP-3 level was lower in patients with oral squamous cell carcinoma than in healthy controls, suggesting that plasma TIMP-3 is a potential biomarker for predicting oral squamous cell carcinoma[12]. These findings, together with our study, indicate that decreased TIMP-3 expression may contribute to procarcinogenic effect.

Indeed, we further evidenced that overexpression of TIMP-3 potentiated cisplatin-induced apoptosis in cisplatin-resistant laryngeal carcinoma cells, while inhibition of TIMP-3 attenuated the sensitivity of laryngeal carcinoma cells to cisplatin. This concurs with discoveries that also show an inhibitory effect of TIMP-3 upregulation on proliferation, migration, invasion, and metastasis in osteosarcoma and chondrosarcoma[10,11]. In addition, we also found that TIMP-3-mediated chemoresistance was associated with the mitochondrial pathway of apoptosis. Forced TIMP-3 expression enhanced, whereas knockdown of TIMP-3 inhibited, cisplatin-induced mitochondrial cytochrome c release. It has been shown that cytochrome c release could result in combination with caspase 9 to form a complex, leading to activation of caspase 3, thus triggering apoptosis[14]. Our data showed that TIMP-3 overexpression further augmented the activation of caspase 9 and caspase 3 induced by cisplatin. However, downregulation of TIMP-3 produced the opposite effects. Collectively, these results suggest that the effect of TIMP-3 on cisplatin resistance of laryngeal carcinoma is partially mitochondria dependent.

In summary, the present study demonstrates that the expression of TIMP-3 may be critical for chemoresistance of laryngeal carcinoma. Elevation of TIMP-3 expression promotes cisplatin-induced activation of the mitochondrial pathway of apoptosis, increasing the sensitivity of laryngeal carcinoma cells to cisplatin therapy. These findings provide important clues to the mechanisms involved in cisplatin resistance of laryngeal carcinoma and suggest that TIMP-3 may be a useful target to improve chemotherapeutic results.

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