Low expression of TUG1 promotes cisplatin sensitivity in cervical cancer by activating the MAPK pathway

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Summary

Purpose: This study aims to investigate whether TUG1 can regulate cisplatin resistance in the disease progression of cervical cancer (CC) by activating the MAPK pathway.

Methods: Taurine-upregulated gene 1 (TUG 1) expression in CC tissues and cell lines was determined by quantitative real-time polymerase chain reaction (qRT-PCR). The correlation between TUG1 expression and the prognosis of CC patients was analyzed by Kaplan-Meier method. The regulatory effects of TUG1 on proliferative and apoptotic rates of DDP-induced CC cells were assessed by cell counting kit-8 (CCK-8), colony formation and TUNEL assay, respectively. The target gene of TUG1 was predicted by online bioinformatics. The expression level of the target gene was determined after TUG1 knockdown. Subsequently, proliferative and apoptotic rates of DDP-induced CC cells with knockdown of the target gene were explored as well. By transfection of shRNA TUG1, the protein expressions of Bcl-2, Bax and relative genes in the MAPK pathway were detected by Western blot.

Results: QRT-PCR showed that TUG1 was highly expressed in CC tissues, especially in those with DDP-resistance. Similarly, TUG1 was highly expressed in CC cell lines as well. Higher expression of TUG1 suggested a worse prognosis of CC patients. TUG1 knockdown inhibited the proliferative rate but accelerated the apoptosis of DDP-induced CC cells. Through bioinformatics prediction, RFX7 was screened out to be the target gene of TUG1. Both mRNA and protein levels of RFX7 were downregulated by TUG1 knockdown. Knockdown of RFX7 could inhibit the proliferative rate and colony formation ability of CC cells. After DDP induction in CC cells, phosphorylated levels of p38 and JNK increased, whereas ERK1/2 expression decreased.

Conclusions: TUG1 is highly expressed in CC tissues and closely related with its DDP-resistance. TUG1 knockdown could inhibit the proliferative rate but accelerate the apoptosis of CC cells through activating the MAPK pathway.

Key words: TUG1, MAPK pathway, cervical cancer, cisplatin

Introduction

Cervical cancer (CC) is a common gynecological malignant tumor. Annually, there are about 190,000 new cases of CC in China. More than 30,000 women die of CC every year, and the incidence age has become younger in recent years [1-3]. Although current preventive and therapeutic measures for CC have greatly developed, the prognosis of advanced CC is far from satisfactory and the overall 5-year survival rate is not high. Moreover, patients with advanced CC are prone to experience tumor recurrence and metastasis [4]. In view of the sensitive chemotherapy of CC, this has been widely applied in clinical treatment.

Cisplatin (cis-Dichlorodiammineplatinum, DDP) is the most common chemotherapy drug in clinical practice. It is a small-molecule platinum compound originally developed to inhibit bacterial growth and that has been identified as a potent anticancer drug. DDP is one of the most effective drugs for the treatment of advanced or recurrent...
CC. Studies have shown that multiple molecular pathways are involved in the anticancer effect of DDP [5]. DDP crosslinks with the purine base of DNA molecules, interferes with DNA repair, and ultimately leads to severe DNA damage. These cellular changes in turn result in the death and necrosis of tumor cells [6]. However, drug resistance seriously influences the therapeutic effect of DDP accompanied by disease progression. A small number of cancer patients have intrinsic resistance to DDP, but more commonly, most cancer patients gradually develop DDP resistance during treatment [7,8]. Highly frequent resistance is the most important factor that limits the therapeutic efficacy of DDP. Moreover, the mechanism of DDP resistance in CC remains unclear.

Long non-coding RNA (lncRNA), a novel non-coding ribonucleic acid (ncRNA), contains more than 200 nucleotides in length. LncRNA was initially considered as a “noise or garbage” in the transcription of the genome since it does not exert biological functions. Compared with microRNA, lncRNA presents structural complexity and diversity of expression regulation. Besides, lncRNA participates in the regulation of protein function, genomic imprinting, transcription and post-transcriptional regulation during transduction [9]. LncRNA provides a novel perspective for the comprehensive interpretation of tumor occurrence and development [9].

LncRNA Taurine-upregulated gene 1 (TUG 1) is widely expressed in human tissue cells and was first found to be highly expressed in the developing retina and nerve tissue. Studies have shown that TUG1 is necessary in for regulating the normal development of retina and nerves [10,11]. Moreover, TUG1 is upregulated in various tumor tissues and cell lines, such as hepatocellular carcinoma, glioma, osteosarcoma, and bladder cancer. Experimental studies have confirmed that TUG1 serves as a proto-oncogene. Overexpression of TUG1 promotes the proliferative and metastatic abilities of tumor cells, but inhibits cell apoptosis [12]. It has been reported that cell apoptosis exerts an essential role in drug resistance. Anti-apoptosis in tumor cells may lead to the failure of tumor treatment [13,14]. However, current research on the anti-apoptotic mechanism of TUG1 is still unclear. Further studies are required in order to investigate whether TUG1 is related to the development of DDP resistance in CC.

**Methods**

**Collection of clinical samples**

Patients who underwent hysterectomy for CC at the Obstetrics and Gynecology Hospital of Fudan University from 2014 to 2016 were enrolled. CC patients who were pathologically diagnosed and did not receive preoperative tumor therapy or biotherapy met our inclusion criteria. Paracancerous tissues that were 2 cm away from the tumor lesions were harvested, and then further confirmed by pathology analysis. All samples were quickly placed in RNA later protection solution, frozen at -80°C in a refrigerator and finally transferred to liquid nitrogen. The experimental procedures were approved by the Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University.

**Cell culture**

Human CC cell lines SiHa, Me180, HCE1, HeLa and CaSki, as well as the normal cervical cell line H8 were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and maintained in a 5% CO₂ incubator at 37°C.

**DDP induction**

HeLa and CaSki cells were seeded in 24-well plates with 4×10⁵ cells per well and incubated overnight. The next day, the culture medium containing different concentrations of DDP (0-400 μM) was replaced for 24-h cell culture.

**Cell transfection**

HeLa and CaSki cells in logarithmic growth phase were digested for cell plating. Until 30-50% of confluence, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or shRNA (Ribobio, Guangzhou, China) was diluted in culture medium and maintained for 5 min. After gentle mixture and maintenance for 20 min at room temperature, the mixture solution was added to the plate. Fresh medium was replaced 4-6 h after transfection.

**RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reversely transcribed into cDNA using the First-strand cDNA Synthesis Kit (TaKaRa, Otsu, Japan). The complementary DNA (cDNA) was amplified using the Fast Start Universal SYBR Green at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. GAPDH was used as loading control. The primers used in this study were as follows: RFX7: F: CATGAGGGCACTGCTTTGG; R: ACGGTGTCAAAATG-GTTGAGCT. TUG1: F: CTATACCTCGTTCAGGT; R: TACTGTATGGCCACCACTCC.

**TUNEL assay**

Cells were incubated with Proteinase K at room temperature for 30 min. After cell wash with phosphate buffered saline (PBS), cells were blocked for 10 min at room temperature and incubated with TdT solution at 37°C for 60 min in the dark. Apoptotic cells were observed and calculated in 5 randomly selected fields of each sample (magnification 40×).
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Cell counting kit-8 (CCK-8)

Cells were seeded in 96-well plates with 1000 cells per well. After cell culture for 0 h, 24 h, 48 h and 72 h, respectively, CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well. Two hours later, the optical density (OD) value of each well was measured at the wavelength of 450 nm using a microplate reader.

Western blot

Protein samples were separated by 10% polyacrylamide gel electrophoresis. After transfer to PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA, USA), the protein-containing membrane was blocked with 5% skim milk for 2 h. The corresponding primary antibody was added and incubated with proteins at 4°C overnight, followed by incubation of the secondary antibody for another 1 h. The ECL (enhanced chemiluminescence) luminescent agent (Thermo Fisher Scientific, Waltham, MA, USA) was used to develop the protein imprint. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Colony formation assay

Cells in the 6-well plate were cultured for 2 weeks in complete medium. The culture medium was replaced once in the first week and twice in the second week. Until colony formation, cells were washed with PBS twice and fixed with 4% paraformaldehyde for 30 min. Cells were then washed with PBS and stained with 0.1% crystal violet staining solution for 20 min. Finally, colonies were observed and captured using a microscope.

Statistics

SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data processing and analysis. The data were expressed as mean ± SD. The two-paired t-test was used to compare the mean values of two independent samples. Survival analysis was conducted by Kaplan-Meier method and the difference was compared using the Log-rank test. P<0.05 was considered statistically significant. All experiments were independently performed in triplicate.

Results

TUG1 was highly expressed in DDP-resistant CC

We firstly examined the expression level of TUG1 in CC patients by RT-PCR. The results showed a higher expression of TUG1 in CC tissues than in paracancer tissues (Figure 1A). According to the median expression of TUG1, the enrolled CC patients were divided into high-expression and low-expression groups. Survival analysis showed that CC patients with higher expression had markedly worse prognosis than patients with lower expression (Figure 1B). To further explore the correlation between TUG1 expression and DDP resistance, we divided CC patients into DDP-resistant and DDP-sensitive groups. A higher expression of TUG1 was observed in DDP-resistant CC patients than in DDP-sensitive CC patients (Figure 1C). Identifi-
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TUG1 was highly expressed in CC cell lines compared with controls (Figure 1D). Meanwhile, DDP-resistant HeLa cells and DDP-resistant CaSki cells expressed higher levels of TUG1 than DDP-sensitive cells (Figure 1E).

**TUG1 knockdown inhibited proliferative rate but accelerated apoptotic rate of DDP-induced CC cells**

To validate the in vitro biological function of TUG1, we used shRNA transfection technology to downregulate TUG1 expression in CC cells (Figure 2A). HeLa and CaSki cells were induced with different concentrations of DDP (0-400 μM) for 24 h. CCK-8 results showed that the viability of CC cells gradually decreased as the DDP concentration increased. Besides, TUG1 knockdown markedly decreased the viability of DDP-induced CC cells (Figure 2B). We selected 50 μM as the DDP concentration used in the following experiments. As our results indicated, cell viability in 50 μM DDP-induced CC cells was gradually inhibited in a time-dependent manner. CC cells transfected with shRNA TUG1 had lower cell viability than controls after induction of 50 μM DDP (Figure 2C). Cell colony formation assay also demonstrated that TUG1 knockdown could inhibit the proliferative rate of DDP-induced CC cells (Figure 2D). TUNEL assay was then conducted to detect apoptosis in DDP-induced cells. A higher apoptotic rate was observed in CC cells with TUG1 knockdown (Figure 2E). Moreover, TUG1 knockdown upregulated Bax expression but downregulated Bcl-2 expression in DDP-induced CC cells (Figure 2F).

**TUG1 promoted RFX7 expression in DDP-induced CC cells**

Through bioinformatics prediction, RFX7 was screened out to be the target gene of TUG1. Here, we determined mRNA and protein levels of RFX7 in DDP-induced CC cells by qRT-PCR and Western blot, respectively. Both mRNA and protein levels of RFX7 were downregulated by TUG1 knockdown

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**Figure 2.** TUG1 knockdown inhibited the proliferative rate but accelerated the apoptotic rate of DDP-induced CC cells. A: TUG1 expression in HeLa and CaSki cells transfected with shRNA-NC or shRNA-TUG1 was verified by qRT-PCR. B: CCK-8 results showed that TUG1 knockdown markedly decreased the viability of HeLa and CaSki cells induced with different concentrations of DDP (0-400 μM) for 24 h. C: CCK-8 results showed the decreased viability of HeLa and CaSki cells induced with 50 μM DDP for 24 h. D: Colony formation assay showed the decreased proliferation of HeLa and CaSki cells induced with 50 μM DDP for 24 h. E: TUNEL staining showed accelerated apoptosis in HeLa and CaSki cells induced with 50 μM DDP for 24 h (magnification 40 ×). F: Protein expressions of Bcl-2 and Bax in HeLa and CaSki cells induced with 50 μM DDP for 24 h by Western blot (*p<0.05).
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Figure 3. TUG1 promoted RFX7 expression in DDP-induced CC cells. A: The mRNA level of RFX7 was downregulated by TUG1 knockdown. B: The protein level of RFX7 was downregulated by TUG1 knockdown. C: CCK-8 results showed that knockdown of RFX7 could inhibit the proliferative rate of HeLa and CaSki cells induced with 50 μM DDP for 24 h. D: Colony formation assay showed that the knockdown of RFX7 could inhibit the proliferative rate of HeLa and CaSki cells induced with 50 μM DDP for 24 h (*p<0.05).

Figure 4. TUG1 knockdown activated the MAPK pathway. A: DDP induction in HeLa cells for 48 h upregulated the phosphorylated levels of p38 and JNK, whereas it downregulated ERK1/2 expression. B: DDP induction in CaSki cells for 48 h upregulated the phosphorylated levels of p38 and JNK, whereas it downregulated ERK1/2 expression (*p<0.05).
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TUG1 knockdown activated MAPK pathway

Previous research pointed out the remarkable role of the MAPK pathway in cell apoptosis. Hence, we speculated that TUG1 knockdown may promote the apoptosis of CC cells through activating the MAPK pathway. DDP induction in CC cells upregulated phosphorylated levels of p38 and JNK, whereas it downregulated ERK1/2 expression (Figure 4A and 4B). We confirmed that TUG1 knockdown markedly activated the MAPK pathway in CC cells.

Discussion

As essential members of ncRNA, IncRNAs are abnormally expressed in various tumors. Changes in expression levels or even spatial structures of IncRNAs could result in a series of changes in protein expressions and signaling pathways, which are the basic conditions of tumor development. Recent studies have proved that IncRNAs are closely related to drug resistance in tumor treatment [15,16]. Therefore, analyses of the molecular mechanisms of IncRNAs related to DDP resistance provide new ideas for developing therapeutic approaches in CC. In this study, we found that TUG1 expression was elevated in CC patients, especially in those with DDP resistance, suggesting the potential role of TUG1 in DDP resistance. Subsequent in vitro experiments showed that DDP could markedly upregulate TUG1 expression. TUG1 knockdown inhibited the proliferative rate but accelerated the apoptosis of CC cells. We also confirmed that the MAPK pathway was involved in TUG1-regulated DDP resistance.

Mitogen-activated protein kinase (MAPK) p38 is a crucial family of protein kinases under physiological and pathological conditions. The MAPKp38 family mainly includes four subtypes, namely p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12), and p38δ (MAPK13) [17]. P38 is greatly involved in the regulation of cell proliferation, division, apoptosis and tumorigenesis [18]. It not only has tumor-promoting effects, but it also inhibits tumorigenesis. The controversial effect of p38 mainly depends on original differences in tumor tissues and cells. Highly expressed p38 in human primary cutaneous squamous carcinoma cells, head and neck squamous carcinoma cells (HNSCC), cholangiocarcinoma cells and hepatocarcinoma cells could promote their proliferative, migratory and invasive capacities [19-21]. P38 is lowly expressed in esophageal squamous cell carcinoma. Overexpression of p38 would inhibit its proliferative and invasive rate, increase DDP sensitivity and accelerate cell apoptosis [22]. Our study consistently found that the pro-apoptotic effect of TUG1 in CC may be associated with p38 activation.

Bcl-2 is a major anti-apoptotic factor whose expression level can indirectly indicate the apoptotic level. Bax, the biological function of which is opposite to Bcl-2, can also be used as an apoptotic indicator. In cells with high apoptotic level, Bcl-2 is downregulated and Bax is upregulated. Under normal physiological conditions, Bcl-2 can interact with Bax and inhibit its activity. However, under the stimulation of apoptotic signal, Bax is released from the Bcl-2/Bax complex and enters the mitochondria due to competitive binding of other apoptotic factors or decreased expression of Bcl-2. Eventually, cytochrome c, the apoptotic messenger in the mitochondrial membrane space, releases and finally activates the apoptotic process [23]. Changes in the expression levels of the anti-apoptotic factor Bcl-2 and the pro-apoptotic factor Bax are the main molecular mechanisms determining apoptosis. Upregulated Bax, downregulated Bcl-2 and accelerated apoptosis in CC cells are found after radiotherapy [24]. In addition, the induction of apoptosis by cisplatin and dihydroartemisinin in HeLa cells depends on downregulation of Bcl-2 and upregulation of Bax [25,26]. It is reported that p38 could directly regulate Bax expression in HeLa cells, thereby regulating cell apoptosis [27]. However, we were unable to study the interaction between p38 and Bax in this study, which needs to be further explored.

Conclusions

TUG1 is highly expressed in CC tissues and closely related with its DDP-resistance. TUG1 knockdown could inhibit the proliferative rate but accelerate the apoptosis of CC cells through activating the MAPK pathway.

Conflict of interests

The authors declare no conflict of interests.
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References


