Long non-coding RNA tumor suppressor candidate 7 advances chemotherapy sensitivity of endometrial carcinoma through targeted silencing of miR-23b

Chao Shang1, Bin Lang2, Cheng Ngok Ao2 and Lirong Meng2

Abstract
Endometrial carcinoma is the most common malignant tumor of the female genital tract worldwide. TUSC7 (tumor suppressor candidate 7) is an antisense long non-coding RNA and is downregulated and acts as a potential tumor suppressor in several malignant tumors. In this study, the low expression of TUSC7 was confirmed in endometrial carcinoma tissues and was associated with high pathological stages of endometrial carcinoma, which revealed that TUSC7 might be involved in tumorigenesis and progression of endometrial carcinoma. Moreover, the expression of TUSC7 in endometrial carcinoma tissues and cell lines resistant to CDDP and Taxol was lower than that in sensitive endometrial carcinoma tissues and cell lines, which indicated that the TUSC7 expression level was positively correlated with the response of endometrial carcinoma patients to chemotherapy with CDDP and Taxol. TUSC7 upregulation inhibited proliferation, blocked cells at G1 phase, and advanced apoptosis and chemotherapy sensitivity to CDDP and Taxol in HEC1A/CR cell line. Furthermore, miR-23b was upregulated in endometrial carcinoma and negatively correlated with the expression of TUSC7. RNA pull-down assay indicated that TUSC7 could specifically silence the expression of miR-23b in HEC1A/CR cell line; miR-23b was a target gene of TUSC7. MiR-23b upregulation mostly reversed the TUSC7-induced regulatory effects on HEC1A/CR cell line. In summary, long non-coding RNA TUSC7 was underexpressed in endometrial carcinoma, especially in endometrial carcinoma chemotherapy-resistant tissues and cell lines and acted as a potential tumor suppressor gene to inhibit cell growth as well as advance the chemotherapy sensitivity through targeted silencing of miR-23b, which might provide a new therapeutic target to endometrial carcinoma.

Keywords
Endometrial carcinoma, long non-coding RNA, tumor suppressor candidate 7, chemotherapy, microRNA-23b

Introduction
Endometrial carcinoma (EC) is the most common and lethal malignant tumor of the female genital tract worldwide.1 For EC, the surgical operation is the preferred therapeutic strategy, and adjuvant chemotherapy and radiotherapy could postpone progress and improve survival.2 Despite therapeutic strategies have great progress in recent decades, the prognosis of EC patients is still poor.3 Chemotherapy resistance limited severely the clinical application of chemotherapy. Thus, it is crucial to research the mechanism of chemotherapy resistance and find a novel therapeutic target to improve the poor prognosis of EC.

Long non-coding RNAs (lncRNAs) are a class of endogenous RNAs which have no protein-coding potential. For the past decade, the lncRNAs are the focus of molecular biology, and related studies progress rapidly.

1Department of Neurobiology, China Medical University, Shenyang, P.R. China
2School of Health Sciences, Macao Polytechnic Institute, Macao, P.R. China

Corresponding author:
Lirong Meng, School of Health Sciences, Macao Polytechnic Institute, R. de Luis Gonzaga Gomes, Macao 000000, P.R. China.
Email: lrmeng@ipm.edu.mo
LncRNAs can regulate their downstream genes and take part in nearly all cellular biological phenotypes, such as cell cycle, apoptosis, autophagy, and drug resistance.\textsuperscript{4–7} Recent literatures reported that aberrant lncRNA expression is observed in almost all malignant human tumors and is involved in tumorigenesis by acting as oncogenes or tumor suppressor genes.\textsuperscript{8–10}

Tumor suppressor candidate 7 (TUSC7) is an antisense RNA gene consisted of four exons and located at 3q13.3.\textsuperscript{11} TUSC7 has no protein-coding potential and belongs to lncRNAs. Recent studies found that TUSC7 was downregulated and acted as a potential tumor suppressor in several malignant tumors, including colorectal cancer, non-small-cell lung cancer, hepatocellular carcinoma, gastric cancer, and so on.\textsuperscript{12–16} Our previous study reported that TUSC7 was underexpressed in human brain glioma and acted as a tumor-suppressing gene to inhibit the malignant behaviors of glioma cell line.\textsuperscript{17} Nevertheless, nothing is known about the expression level and functional roles of TUSC7 in EC, especially its regulatory effect on chemotherapy sensitivity.

In this study, the expression level of TUSC7 in EC was examined, and its regulatory effects and mechanisms on chemotherapy sensitivity in EC were studied. The findings of our study will contribute to the development of effective clinical interventions targeting EC.

**Materials and methods**

**Clinical specimens**

In total, 58 EC and 31 normal endometrial tissue (NET) specimens were obtained from the Department of Gynaecology in Shengjing Hospital through hysteroscopy from September 2014 to February 2016. This study was approved by the Ethics Committees of Macao Polytechnic Institute, and all patients’ written consents were obtained. All 58 EC patients were treated by chemotherapy with cisplatin (CDDP) and paclitaxel (Taxol) and divided into two groups according to therapeutic effects. EC-sensitive patients (n=23) were relieved completely or partially, and the condition of resistant patients (n=35) were stable or deteriorating. The clinicopathological data were affirmed by professional pathologist.

**Quantitative real-time polymerase chain reaction**

The total RNA was extracted from EC tissues and cells, and the SYBR (#4367659; Applied Biosystems, Foster City, CA, USA) was used to detect the expression of TUSC7 according to manufacturer’s instructions. The forward primer of TUSC7 was 5′-TTTATGCTTGAGCCTTG A-3′ and the reverse primer was 5′-CTTGCCCTGAAATAC TTGC-3′. The relative expression of TUSC7 was quantified by 2\textsuperscript{−ΔΔCt} method when normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Cell culture**

Human normal endometrial epithelial cell line (ESC) and EC cell line (HEC1A) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C. The chemotherapy-resistant HEC1A/CR cell line (resistant to CDDP and Taxol) was gifted from Department of Gynaecology, Shengjing Hospital.

**Vectors construction and transfection**

The expression vector pE-TUSC7 and negative control vector pE-NC were constructed by GenScript (Nanjing, China). MiR-23b agonist (agomir-23b) and agomir-NC were achieved from GenePharma (Shanghai, China). The vectors and microRNAs were transfected respectively into HEC1A/CR cell line by Lipofectamine 3000 (Invitrogen, Foster City, CA, USA) according to manufacturer’s instructions. The stable silence cell lines were established by G418 (Invitrogen) culture, and the transfection effect was examined by quantitative real-time polymerase chain reaction (qRT-PCR).

**Cell proliferation assay**

The cells (2 × 10\textsuperscript{4}/well) were seeded on 96-well plate. A volume of 10 µL of Cell Counting Kit-8 (CCK-8; Beyotime, Jiangsu, China) was added into each well and incubated for 2h at 37°C. Absorbance at 450 nm was recorded using the SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

**Flow cytometry assay**

For the cell cycle assay, 1 × 10\textsuperscript{6} cells were collected, fixed, and stained with propidium iodide (PI); the cell cycle was determined by the flow cytometry (FACScan; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

The apoptosis detection was carried out with Annexin V–fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Biosea, Beijing, China) according to manufacturer’s instructions and analyzed using CELLQuest software (Becton, Dickinson and Company). Cells in the right lower quadrant were regarded as apoptotic.

**Chemoresistance assay**

Cells were seeded onto 96-well plates with 3000 cells per well and treated with CDDP (10, 50, 100, 150, and 200 µg/mL) or Taxol (10, 20, 50, 100, and 150 µg/mL) 24 h later.\textsuperscript{18,19} After 48h, the cellular viability was detected, and the
inhibition rate for every concentration and the half maximal inhibitory concentration (IC$_{50}$) were calculated using GraphPad5 software (Graphpad Software, La Jolla, CA).

**RNA pull-down assay**

Probes for TUSC7 or miR-23b were biotinylated (Sangon, Shanghai, China) and transfected into HEC1A/CR cell line. After 48h, the cells were harvested and lysed. The samples were incubated with Dynabeads M-280 Streptavidin (Invitrogen). Biotinylated miR-23b was incubated with beads for 10 min and treated with washing buffer. The bound RNAs were analyzed by qRT-PCR.

**Statistical analysis**

All data were showed as mean ± standard error of the mean (SEM) of five independent experiments and analyzed with GraphPad Prism 5.0 (Graphpad Software). For comparing the differences between them, paired Student’s t test and one-way analysis of variance (ANOVA) were used. The correlation between TUSC7 expression and pathologic data was analyzed by one-way ANOVA and binary logistic regression. $p < 0.05$ was considered to be statistically significant.

**Results**

**The expression level and clinical significance of TUSC7 in EC**

In this study, the qRT-PCR was used to investigate the TUSC7 expression level in EC tissues and HEC1A cell line compared with control NET tissues and ESC cell line. The results showed that the TUSC7 expression in EC specimens was lower than that in control NET specimens (Figure 1(a), $p<0.05$), and the TUSC7 expression in HEC1A cell line was also lower than that in ESC cell line (Figure 1(b), $p<0.05$).

In addition, the correlation between TUSC7 expression and pathologic data of EC was investigated. The expression of TUSC7 in tumor lesion was negatively correlated with
pathological stages (International Federation of Gynecology and Obstetrics (FIGO) 2009). With the increase of pathological stage, the expression of TUSC7 was downregulated (Figure 1(c), \( p < 0.05 \)). But TUSC7 expression was not significantly correlated with other clinical characteristics, including age, invasive depth, and lymph node metastasis \( (p > 0.05) \). These findings provided initial evidence that aberrant expression of TUSC7 might be involved in tumorigenesis and progression of EC but not in metastasis.

**Low expression of TUSC7 was related to chemotherapy resistance of EC**

Meanwhile, the expression level of TUSC7 in EC patients was analyzed further. The qRT-PCR results showed that the expression of TUSC7 in resistant EC patients was much lower than that in sensitive patients (Figure 2(a), \( p < 0.05 \)), which provided initial evidence that TUSC7 plays a key role in the chemotherapy resistance of EC.

In order to verify the above conjecture, the chemotherapy-resistant HEC1A/CR cell line was established and TUSC7 expression was detected. As shown in Figure 2(b) and (c), the IC\(_{50}\) of CDDP and Taxol in HEC1A cell line was 41.52 and 21.38 µg/mL, respectively, and that in HEC1A/CR cell line was 153.26 and 82.75 µg/mL, respectively \( (p < 0.05) \). The HEC1A/CR cell line had high resistance to CDDP and Taxol \( (p < 0.05) \), and their resistant index (RI) were 3.69 and 3.87. Compared with HEC1A cell line, the TUSC7 expression was much lower in HEC1A/CR cell line (Figure 2(d), \( p < 0.05 \)).

These findings exposed that the low expression of TUSC7 was positively correlated with the response of EC patients to chemotherapy with CDDP and Taxol.

**Upregulation of TUSC7 inhibited proliferation and advanced apoptosis of HEC1A/CR cell line**

To validate whether TUSC7 affected the functional roles of HEC1A/CR cell line, the pE-TUSC7 was transfected into HEC1A/CR cell line to upregulate the expression of TUSC7 (Figure 3(a), \( p < 0.05 \)).

First, the CCK-8 assay discovered that the upregulation of TUSC7 depressed cell viability of HEC1A/CR cell line (Figure 3(b), \( p < 0.05 \)). Second, PI single–labeled flow cytometry found that HEC1A/CR cell line with TUSC7 upregulation showed a significant G1 phase block (Figure 3(c), \( p < 0.05 \)). Third, the apoptosis rate of HEC1A/CR cell line was advanced by TUSC7 upregulation as detected by Annexin V–FITC double–labeled flow cytometry (Figure 3(d), \( p < 0.05 \)).

Together with the above findings, TUSC7 upregulation inhibited proliferation and advanced apoptosis and participates in cell growth of HEC1A/CR cell line.

**Upregulation of TUSC7 advanced chemotherapy sensitivity of HEC1A/CR cell line**

Chemotherapy with CDDP and Taxol is one of the first-line chemotherapy schemes for EC patients. In HEC1A/CR cell line, upregulation of TUSC7 restrained the IC\(_{50}\) of
CDDP from 161.14 to 69.35 µg/mL (Figure 4(a), \( p < 0.05 \)); the IC\(_{50}\) of Taxol also decreased from 80.71 to 33.26 µg/mL (Figure 4(b), \( p < 0.05 \)). TUSC7 upregulation advanced chemotherapy sensitivity of HEC1A/CR cell line significantly, which revealed that TUSC7 was involved in the genesis of chemotherapy resistance.

**TUSC7 specifically silenced the expression of miR-23b in HEC1A/CR cell line**

The potential targeted microRNAs of TUSC7 were forecasted by bioinformatics analysis software, including Starbase and TargetScan. A target combination between TUSC7 and miR-23b was found at the 1125–1140 bp of TUSC7 transcript (Figure 5(a)). The qRT-PCR assays found that miR-23b was upregulated significantly in EC specimens than that in control NET specimens (Figure 5(b), \( p < 0.05 \)), and the expression of miR-23b in resistant EC patients was also much higher than that in sensitive patients (Figure 5(c), \( p < 0.05 \)). The Pearson’s correlation analysis showed a negative correlation between the expression of TUSC7 and miR-23b in EC and control NET specimens (\( r = -0.1112 \), \( p = 0.0112 \)).

As shown in Figure 5(d), the upregulation of TUSC7 significantly silenced the expression of miR-23b in HEC1A/CR cell line (\( p < 0.05 \)). Moreover, the RNA pull-down assay showed that TUSC7 was pulled down by biotinylated miR-23b probe (Figure 5(e)) but not by biotinylated miR-23b-mut containing mutations in the putative binding site. On the contrary, the biotin-labeled TUSC7 probe was
used to demonstrate that TUSC7 was also pulled down by miR-23b (Figure 5(f)).

To sum up, TUSC7 could silence directly and specifically the expression of miR-23b in HEC1A/CR cell line; miR-23b was a target gene of TUSC7.

**Upregulation of miR-23b mostly reversed TUSC7-induced regulatory effects**

In this study, agomir-23b was transfected into HEC1A/CR cell line to increase miR-23b expression which was silenced by TUSC7 upregulation. After transfection, the proliferation, apoptosis, and chemotherapy sensitivity of HEC1A/CR cell line were examined.

Results showed that miR-23b upregulation mostly reversed the TUSC7-induced regulatory effects on HEC1A/CR cell line, including promoting the cell proliferation and division and depressing cell apoptosis (Figure 3(b)–(d), \( p < 0.05 \)).

The upregulation of miR-23b also, mostly, reversed the chemotherapy sensitivity of HEC1A/CR cell line advanced by TUSC7 upregulation. The IC\(_{50}\) of CDDP and Taxol in HEC1A/CR cell line was increased from 69.35 and 33.26 µg/mL to 124.16 and 59.58 µg/mL, respectively (Figure 4(a) and (b), \( p < 0.05 \)).

**Discussion**

LncRNA TUSC7 was first identified in osteosarcoma by Pasic et al.\(^{11}\) in 2010. Thereafter, several literatures reported that TUSC7 was downregulated and acted as a potential tumor suppressor in some malignant tumors, including colorectal cancer, non-small-cell lung cancer, hepatocellular carcinoma, gastric cancer, and so on.\(^{12-16}\) Our previous study found that TUSC7 was underexpressed in human brain glioma and acted as a tumor-suppressing gene to inhibit the malignant behaviors of glioma cell lines.\(^{17}\) Nevertheless, nothing is known about the expression level and functional roles of TUSC7 in gynecologic tumor, including EC.

In this study, the low expression of TUSC7 was confirmed in EC tissues, and its low expression was associated with high pathological stages of EC but not with age, invasive depth, and lymph node metastasis. These outcomes indicated that TUSC7 might be involved in the genesis and progression of EC but not in metastasis of EC.

Recently, altered expression of some lncRNAs reported to contribute to chemotherapy resistance of human tumors, which provided novel targets to clinical therapy of tumors. For instance, silencing the Cancer Susceptibility Candidate 9 advanced the chemotherapy sensitivity of gastric cancer-resistant cell lines to Taxol and Adriamycin.\(^{20}\) Jiang et al.\(^{21}\) found that high taurine upregulated gene 1 (TUG1) expression was significantly correlated with chemotherapy resistance of esophageal squamous cell carcinoma, and patients with high TUG1 expression had poor prognosis. Till now, none of lncRNAs was reported to be involved in the chemotherapy resistance of EC.

Our study found that the expression of TUSC7 in EC tissues and cell lines resistant to CDDP and Taxol was lower than that in sensitive EC tissues and cell lines, which indicated that the TUSC7 expression level was positively correlated with the response of EC patients to chemotherapy with CDDP and Taxol. After that, TUSC7 was upregulated to examine its regulatory roles in EC chemotherapy-resistant cell line. The results showed that TUSC7 upregulation significantly advanced the chemotherapy sensitivity of HEC1A/CR cell line to CDDP and Taxol. Moreover, TUSC7 upregulation inhibited the proliferation, blocked cells at G1 phase, and advanced apoptosis of HEC1A/CR cell line. These findings indicated that TUSC7 could inhibit cell growth as well as advance chemotherapy sensitivity and proved that TUSC7 acted as a potential tumor suppressor gene in EC. However, the molecular mechanism in this regulatory process is still unknown.

Bioinformatics analysis was used to predict the potential target genes of TUSC7, a specific combination between...
TUSC7 and miR-23b was found. Our previous study had validated this combination in glioma cell line where TUSC7 downregulated the expression of miR-23b.17 In order to confirm whether the above regulatory effect also exists in EC cell, a series of assays are used to test this hypothesis. First, the miR-23b expression was upregulated significantly in EC specimens and showed a negative correlation with the TUSC7 expression. Second, the upregulation of TUSC7 significantly silenced the expression of miR-23b in HEC1A/CR cell line. Third, RNA pull-down assay confirmed the direct and specific combination between TUSC7 and miR-23b. To sum up, TUSC7 could specifically silence the expression of miR-23b in HEC1A/CR cell line; miR-23b was a target gene of TUSC7.

Furthermore, we investigated whether miR-23b mediated the regulatory effects induced by TUSC7 upregulation on HEC1A/CR cell line. Our data indicated that miR-23b upregulation mostly reversed the TUSC7-induced regulatory effects on HEC1A/CR cell line, including advancing the cell proliferation and division and depressing the apoptosis and chemotherapy sensitivity. Therefore, TUSC7 could inhibit cell proliferation and division as well as advance apoptosis and chemotherapy sensitivity through targeted silencing of miR-23b.

In conclusion, lncRNA TUSC7 was underexpressed in EC, especially in EC chemotherapy-resistant tissues and cell line, and acted as a potential tumor suppressor gene to inhibit cell growth as well as advance chemotherapy sensitivity through targeted silencing of miR-23b. These achievements might provide a novel therapeutic target for clinical treatment of EC.

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