Abstract. – OBJECTIVE: The aim of the present study was to explore the prognostic value of long non-coding RNA CPS1-IT1 (CPS1-IT1) expression in epithelial ovarian cancer (EOC) patients and identify the effect of CPS1-IT1 on cell proliferation and apoptosis of EOC cells.

PATIENTS AND METHODS: Expression levels of CPS1-IT1 in tissues and cells were detected by the Real-time quantitative RT-PCR assay. The χ²-test was used to analyze the relationship between CPS1-IT1 expression and the clinicopathological characteristics. Survival analysis was performed using the Kaplan-Meier method and Cox's proportional hazards model. The capacity for cellular proliferation was measured with cell counting Kit-8. Cell apoptosis assays were performed using flow cytometry. Western blot was used to detect the expression levels of cell apoptosis-related proteins.

RESULTS: We observed that CPS1-IT1 was significantly downregulated in EOC cell lines and tissue samples. The expression of CPS1-IT1 was significantly associated with FIGO stage and lymph node metastases. In addition, EOC patients in the low tissue CPS1-IT1 expression group had significantly shorter 5-year overall survival time than those in the high tissue CPS1-IT1 expression group. Furthermore, univariate and multivariable Cox regression analysis identified low CPS1-IT1 expression in EOC tissues as an independent poor prognostic marker of overall survival. It was also found that over-expression of CPS1-IT1 markedly promoted proliferation of EOC cells. Further studies revealed that over-expression of CPS1-IT1 induced cell apoptosis by through regulating apoptosis-related proteins.

CONCLUSIONS: CPS1-IT1 may be a functional tumor suppressor in EOC. It may also serve as an independent prognostic factor for patients with EOC.

Key Words: Long non-coding RNA CPS1-IT1, Proliferation, Apoptosis, Prognosis, Epithelial ovarian cancer.
In the current study, we firstly determined the expression levels of CPS1-IT1 in EOC patients. Additionally, the correlation of CPS1-IT1 levels with clinicopathological factors and prognosis was also statistically analyzed. Moreover, the role of CPS1-IT1 overexpression in EOC cells was studied.

Patients and Methods

Patients
A total of 91 EOC samples and matched noncancerous tissue samples were randomly obtained from samples that were removed from patients who were treated in the People’s Hospital of Linyi City between 2010 and 2012. The fresh tissue samples were immediately snap-frozen in liquid nitrogen. None of these patients had received radiotherapy or chemotherapy prior to surgery. The histopathological type and stage of the EOC were determined according to the criteria of the World Health Organization classification. The study was approved by the Ethics Committee of People’s Hospital of Linyi City and was conducted in compliance with the Helsinki Declaration. Informed consent was obtained from every patient.

Cell Culture and Recombinant Plasmid Transfection
The human EOC cell lines (A2780, CAOV-3, and ES-2) and normal human ovarian surface epithelial (HOSE) cells were supplied by China Center for Type Culture Collection (CCTCC). All cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) GIBCO (Rockville, MD, USA) in a humidified atmosphere of 5% CO₂ at 37°C. In this study, plv-MVC-vector plasmids and plv-MVC-CPS1-IT1 plasmids were purchased from Ambion (Austin, TX, USA). A2780 cells were transfected with either plv-MVC-vector plasmids and plv-MVC-CPS1-IT1 plasmids by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

RNA Extraction and qRT-PCR
Total RNA was extracted from tissues and cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA). RNA was reverse transcribed into cDNAs using a Prime-Script™ RT-PCR kit (TaKaRa, Dalian, China). Expressions of IncRNA were detected using ABI 7300 system (Applied Biosystems, Foster City, CA, USA). GAPDH was employed to be the control. The primers used for target genes were purchased from Invitrogen (Carlsbad, CA, USA).

Cell Proliferation Assay
The cell proliferation assay was performed using the Cell Counting Kit-8 (CCK8) solution (Dojindo, Gaithersburg, MD, USA), according to the manufacturer’s protocol. Cells (2×10⁴/well) were seeded in 96-well plates and adhered overnight. After 48 h of transfection, CCK-8 solution was added to each well and incubated for 1 h at 37°C. The absorbance of the solution was measured spectrophotometrically at 450 nm with an MRX II absorbance reader (Dynex Technologies, Chantilly, VA, USA).

Cell Cycle Analysis
To investigate the role of CPS1-IT1 in the apoptosis of EOC cells, CPS1-IT1 was over-expressed in A2780 cells via transfection with plv-MVC-CPS1-IT1 plasmids. Then, cells were harvested and the apoptotic cells were evaluated using the FITC-Annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions.

Western Blot Assay
Cells were collected and lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). Proteins (20 μg per lane) were separated by 12% sodium dodecyl sulphate-polycrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk in Tris Buffered saline Tween (TBST) and incubated with the antibody against caspase-3 (1:1000), caspase-8 (1:2000), caspase-9 (1:1000), Bax (1:1500) and Bcl-2 (1:1000). Then, membranes were incubated with secondary antibodies (1:1000) conjugated with horse-radish peroxidase (HRP) at RT for 50 min. The protein bands were quantitated by densitometry using the gel analysis software ImageJ (National Institutes of Health, Bethesda, MA, USA). The experiment was conducted in triplicate.

Statistical Analysis
Statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA, USA) and the SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). The significance of differences between groups was estimated by Student’s t-test, χ² test or Wilcoxon test, as appropriate. Survival
curves were constructed with the Kaplan-Meier method and compared by log-rank tests. The survival data were evaluated by univariate and multivariate Cox regression analyses. \( p < 0.05 \) was considered statistically significant.

**Results**

**CPS1-IT1 is Significantly Downregulated in EOC Tissues and Cell Lines**

LncRNA expression levels were examined by Real-time PCR in three EOC cell lines and in 91 paired human EOC and matched adjacent non-tumor tissues. As shown in Figure 1A, the results showed that the expression level of CPS1-IT1 cancer tissues decreased significantly compared to their matched non-tumor tissues (\( p < 0.01 \)). Similarly, we also found that the relative expression of CPS1-IT13 in EOC cell lines was significantly lower than that in the normal cell lines (\( p < 0.05 \), Figure 1B).

**Relationship Between CPS1-IT1 and Clinicopathological Characteristics of EOC Patients**

In order to explore the association between CPS1-IT expression and clinicopathologic features in EOC, we manually divided EOC patients into two groups (high group and low group) based on the mean expression of CPS1-IT

<table>
<thead>
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<th>Variables</th>
<th>CPS1-IT1 expression level</th>
<th>( p )-value</th>
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<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td>0.466</td>
</tr>
<tr>
<td>&lt; 55</td>
<td>43</td>
<td>20</td>
</tr>
<tr>
<td>≥ 55</td>
<td>48</td>
<td>26</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>I-II</td>
<td>61</td>
<td>25</td>
</tr>
<tr>
<td>III-IV</td>
<td>30</td>
<td>21</td>
</tr>
<tr>
<td>Histology</td>
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<td>0.884</td>
</tr>
<tr>
<td>Serous</td>
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<td>30</td>
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<td>Nonserous</td>
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</tr>
<tr>
<td>Grade</td>
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</tr>
<tr>
<td>Well + Moderate</td>
<td>63</td>
<td>28</td>
</tr>
<tr>
<td>Poor</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>Negative</td>
<td>63</td>
<td>26</td>
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<tr>
<td>Positive</td>
<td>28</td>
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in EOC. As shown in Table I, we observed that the expression of CPS1-IT1 was significantly associated with FIGO stage \((p = 0.009)\) and lymph node metastases \((p = 0.008)\). However, the expression levels of CPS1-IT in patients with EOC were not significantly correlated with sex, age, histology, and grade (all \(p < 0.05\)).

**Prognostic Values of CPS1-IT Expression in EOC Patients**

Kaplan-Meier survival curves demonstrated that overall survival was significantly lower in patients with low CPS1-IT level than those with high levels \((p = 0.012; \text{Figure 2})\). Univariate Cox regression analysis showed that overall survival was strongly influenced by FIGO stage \((p = 0.006)\), lymph node metastases \((p = 0.002)\) and CPS1-IT1 expression \((p = 0.004)\) (Table II). Also, multivariate COX regression analysis confirmed that CPS1-IT3 expression levels were independent prognostic factors for overall survival \((HR=2.789, 95\% \ CI 1.522-4.762, p = 0.006)\).

**Up-regulation of CPS1-IT Suppressed Cell Growth in Human EOC Cell Lines**

Our previous results indicated that CPS1-IT may have a negative effect on the proliferation of EOC cells. To confirm the effects of CPS1-IT on EOC cell proliferation, we transfected plv-MVC-CPS1-IT1 plasmids into A2780 cells. The increased levels of CPS1-IT1 can be detected in the A2780 cells after transfection (Figure 3A). Then, the cell proliferation changes of EOC cells were determined using both CCK-8 assay. As shown in Figure 3B, the results indicated that plv-MVC-CPS1-IT1 transfection significantly decreased cell growth compared with scrambled plv-MVC transfection in A2780 cells \((p < 0.05)\).

**Up-regulation of CPS1-IT Promotes Cell Apoptosis**

Furthermore, flow cytometric analysis was performed to further assess whether the effect of CPS1-IT on proliferation of EOC cells by affecting cell apoptosis. Our data showed that compared with the control group, the apoptotic rate of A2780 cell line was increased after transfecting them with plv-MVC-CPS1-IT1 plasmids \((p < 0.01, \text{Figure 3C})\). To further determine the mechanism of CPS1-IT on apoptosis, we tested the levels of mitochondrial and caspases dependent pathway-related proteins. As shown in Figure 4A and 4B, the results showed that the expression of caspase-3, caspase-9 and Bax were significantly increased and Bcl-2 was significantly decreased with translation of plv-MVC-CPS1-IT1 plasmids.

### Table II. Univariate and multivariate analysis of overall survival in ovarian cancer patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age</td>
<td>1.231</td>
<td>0.783-1.653</td>
</tr>
<tr>
<td>FIGO stage</td>
<td>3.877</td>
<td>1.783-6.782</td>
</tr>
<tr>
<td>Histology</td>
<td>1.642</td>
<td>0.832-1.642</td>
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<tr>
<td>Grade</td>
<td>1.582</td>
<td>0.793-2.235</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td>4.132</td>
<td>1.933-7.672</td>
</tr>
<tr>
<td>CPS1-IT1 expression</td>
<td>3.257</td>
<td>1.744-5.459</td>
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</tbody>
</table>
Figure 3. Over-expression of CPS1-IT1 inhibited proliferation and induced apoptosis in EOC cells. (A) qRT-PCR determined CPS1-IT1 expression in A2780 cells transfected with pLv-CMV-CPS1-IT1 or pLv-CMV. (B) Cell viability was measured at posttransfection by CCK-8 assay. (C) The apoptosis rate of A2780 cell lines and patient derived cells were assessed by flow cytometry following Annexin V and propidium iodide staining. The results were presented as mean ± SD from three independent experiments. *p < 0.05, **p < 0.01.

Figure 4. The effect of CPS1-IT1 up-regulation on the expression of apoptosis relevant protein. (A) Western blot analysis was performed to determine the levels of caspase 3, caspase 9, Bax and Bcl-2. (B) RT-PCR was used to analyze the mRNA levels of caspase 3, caspase 9, Bax and Bcl-2. *p < 0.05, **p < 0.01.
These results revealed that CPS1-IT1 over-expression induced apoptosis through mitochondrial and caspases dependent pathway.

Discussion

The treatment of EOC remains great challenges. Looking for the novel molecular markers in EOC may have significant effect in improving the current situation of diagnosis and treatment. Studies have also shown that IncRNAs are involved in tumor genesis and cancer progression14. For instance, Nie et al15 found that IncRNA ANRIL was significantly upregulated in lung cancer, and it could promote cell proliferation and inhibit cell apoptosis by silencing KLF2 and P21 expression. Wu et al16 reported that IncRNA MALAT1 might serve as an oncogenic IncRNA that promotes proliferation and metastasis of gallbladder cancer and activates the ERK/MAPK pathway. Ruan et al17 indicated that IncRNA SNHG12 promoted cell proliferation and migration by upregulating angiogatin gene expression in human osteosarcoma cells. Furthermore, some of the IncRNAs are of prognostic significance, indicating a promising role in the EOC tumorigenesis18,19. These studies showed the great potential of IncRNAs as markers for prognostic and therapeutic strategies against EOC.

CPS1-IT1 was firstly identified as a tumor suppressor in hepatocellular carcinoma by Wang et al13. They found that expression of CPS1-IT1 was significantly decreased in hepatocellular carcinoma tissues, and patients with low CPS1-IT1 expression had poor survival outcomes, suggesting that CPS1-IT1 plays a negative regulator in development of hepatocellular carcinoma. In addition, they performed cells experiment and found that CPS1-IT1 significantly reduced cell proliferation, migration and invasion capacities through regulating HIF-1α activity and inhibiting epithelial-mesenchymal transition. These results explained the reason why patients with low CPS1-IT1 expression had poor prognosis. To our best knowledge, this is the only one paper about the role of CPS1-IT1 in tumors. We wondered whether CPS1-IT1 also plays a key role in the development and progression of EOC.

In the present study, we found that CPS1-IT1 was significantly downregulated in EOC cell lines and tissue samples by RT-PCR. Then, we observed that the expression of CPS1-IT1 was significantly associated with FIGO stage and lymph node metastases, suggesting that CPS1-IT1 might be closely related to tumor development and prognosis of EOC. Moreover, the results of Kaplan-Meier method revealed that the 5-year overall survival of low CPS1-IT1 expression group was significantly shorter than that of high CPS1-IT1 expression group. In addition, multivariate analysis confirmed that CPS1-IT1 expression level was independent prognostic factors for overall survival. However, one of limitations was that the sample size was small. In order to reveal the role of CPS1-IT1 in EOC cells, we assessed the effect of CPS1-IT1 on cell proliferation and apoptosis. We found that CPS1-IT1 overexpression inhibited EOC cell proliferation and induced cell apoptosis. In addition, we confirmed that CPS1-IT1 over-expression induced apoptosis through mitochondrial and caspases dependent pathway. Our findings suggested that CPS1-IT1 served as anti-oncogene in EOC. However, the present study has not elucidated the exact molecular mechanisms of CPS1-IT1 acting on EOC. The exploration of the underlying mechanisms of CPS1-IT1 is necessary.

Conclusions

We suggest that CPS1-IT1 may be a new efficient biomarker for prognosis prediction for EOC patients. Further studies are needed to confirm our results and to further explore whether CPS1-IT1 could be a useful target for the treatment of this disease.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

4) Shapira I, Oswald M, Lovecchio J, Khalil H, Menzin A, Whyte J, Dos Santos, L, Liang S, Bhuiya T, Keogh
Role of CPS1-IT1 in EOC


13) WANG TH, YU CC, LIN YS, CHEN TC, YEH CT, LIANG KH, SHIEH TM, CHEN CY, HUH CH. Long noncoding RNA CPS1-IT1 suppresses the metastasis of hepatocellular carcinoma by regulating HIF-1α activity and inhibiting epithelial-mesenchymal transition. Oncotarget 2016; 7: 43588-43603.


