LncRNA DCST1-AS1 Was Upregulated in Endometrial Carcinoma and May Sponge miR-92a-3p to Upregulate Notch1

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Introduction: The functions of DCST1-AS1 have been investigated in liver cancer, while its role in endometrial carcinoma (EC) remains hardly known. This study aimed to analyze the role of DCST1-AS1 in EC.

Methods: Paired EC and non-tumor tissue samples were obtained from 62 EC patients. These patients were followed up for 5 years since their admission to record their survival conditions. HEC-1 cells were transfected with DCST1-AS1, Notch1 vectors, miRNA negative control or miR-92a-3p mimic. Luciferase activity was measured. qPCR and Western blot were applied to determine the RNA level and protein expression, respectively. The invasion and migration of HEC-1 cells were analyzed by Transwell assay.

Results: We in this study found that DCST1-AS1 was upregulated in EC. Survival analysis revealed that high levels of DCST1-AS1 expression predicted poor survival of EC patients. Bioinformatics analysis revealed that miR-92a-3p may bind DCST1-AS1 and the interaction between them was further confirmed by dual-luciferase activity assay. However, overexpression of miR-92a-3p and DCST1-AS1 failed to affect the expression of each other. Moreover, DCST1-AS1 overexpression led to upregulated Notch1 and increased cancer cell invasion and migration rates. Overexpression of miR-92a-3p played an opposite role and attenuated the effects of DCST1-AS1 overexpression.

Discussion: DCST1-AS1 is downregulated in EC and may sponge miR-92a-3p, thereby promoting cancer cell invasion and migration.

Keywords: endometrial carcinoma, DCST1-AS1, miR-92a-3p, Notch1

Introduction

The most common malignancy of the genital tract of females’ endometrial carcinoma (EC) affects about 10 to 20 out of 100,000 people per year. In recent years, the incidence of EC showed an increasing trend and the onset age is becoming younger and younger. With appropriate surgical resection, the overall survival of EC patients diagnosed at the early stages can reach 80%. However, a considerable portion of EC patients are diagnosed at advanced stages and the prognosis is generally poor. Adjuvant therapeutic approaches, such as chemotherapy and radiation therapies are beneficial for short-term survival, while side effects are inevitable and long-term survival is poor. Therefore, novel therapeutic approaches are still needed.

The application of next-generation sequencing technique in clinical studies has identified a considerable number of genetic alterations involved in the pathogenesis
of EC.\textsuperscript{6,7} Analysis of the functions of genetic players in EC may provide novel insights into the design of targeted cancer therapies.\textsuperscript{8,9} Notch signaling is highly conserved in animal cells.\textsuperscript{10} In cancer biology, Notch signaling is dysregulated and plays varied roles in different types of cancers by regulating cancer cell behaviors.\textsuperscript{11} In a recent study, Song, et al reported that miR-92a-3p can target Notch1 to participate in glioma,\textsuperscript{12} while the interaction between miR-92a-3p and Notch1 in other malignancies remains elusive. LncRNA DCST1-AS1 is recently characterized by oncogenic lncRNA in liver cancer.\textsuperscript{12–14} Our bioinformatics analysis showed that DCST1-AS1 may interact with miR-92a-3p. This study was therefore carried out to investigate the interaction between DCST1-AS1 and miR-92a-3p in EC.

\section*{Materials and Methods}

\subsection*{Sample Collections}
Paired EC and non-tumor tissue samples were obtained from 62 EC patients (48 to 69 years; 56.1±6.3 year) who were admitted to The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China hospital between March 2012 and March 2014. This study passed the review of the Ethics Committee of the aforementioned hospital and was carried out in accordance with the principles of the Declaration of Helsinki. The tissue samples were collected through biopsies under the guidance of MRI. All tissue samples were subjected to histopathological exams to make sure the correct tissue samples were obtained. All patients were newly diagnosed EC patients and none of them received any therapies before the initiation of this study. Patients complicated with other clinical disorders were also excluded from this study. All patients signed written informed consent after they were informed of the principle of the experimental design of this study.

\subsection*{Treatment and Follow-Up}
Therapeutic approaches are decided mainly based on clinical stages, which were determined based on the AJCC staging system. Based on this system, 62 patients included 12, 18, 13 and 19 cases at clinical stage I, II, III, and IV, respectively.

All patients were followed up for 5 years since their admission to record their survival conditions. Follow-up was performed in a monthly manner through telephone. All patients completed this follow-up study.

\section*{EC Cells and Transient Transfections}
Cells of human HEC-1 (ATCC, USA) EC cell line were used in this study. Cell culture medium was composed of 90% Dulbecco’s modified Eagle’s medium and 10% FBS. Cell culture conditions were: 5% CO\textsubscript{2}, 37 °C and 95% humidity.

Expression vectors of DCST1-AS1 and Notch1 were constructed using pcDNA3.1 vector (Invitrogen) as the backbone. MiRNA negative control (NC) and miR-92a-3p mimic were synthesized by Invitrogen. HEC-1 cells were harvested at 75–85% confluence and 10-nM vector or 50-nM miRNA was transfected into 10\textsuperscript{6} cells using lipofectamine 3000 (Invitrogen). Cells were harvested at 24 hrs post-transfection to perform the following studies. NC group contained cells with miRNA NC or empty pcDNA3.1 vector transfection. C group included untransfected cells.

\subsection*{Dual Luciferase Reporter Assay}
DCST1-AS1 luciferase reporter vector was constructed using pGL3 vector (Promega Corporation) as the backbone. HEC-1 cells were co-transfected with either the combination of DCST1-AS1 vector + miR-92a-3p (miR-92a-3p group) or the combination of DCST1-AS1 vector + NC miRNA (NC group). Luciferase activity was measured at 48-hrs post-transfection using dual luciferase (firefly-renilla) assay system (Promega Corporation).

\subsection*{RNA Preparation and qPCR Assays}
HigherPurity\textsuperscript{TM} Total RNA Extraction Kit (Canvax Biotech) was used to extract total RNAs from 10\textsuperscript{5} HEC-1 cells or 0.05 g tissue samples. All RNA samples were digested with gDNA eraser (Takara) and RNA concentrations were measured using NanoDrop\textsuperscript{™} 2000c Spectrophotometer (Thermo Fisher Scientific). SSRT IV (Thermo Fisher Scientific) was used to reverse transcribe total RNAs into cDNA and QuantiTect SYBR Green RT-PCR Kit (QIAGEN) was used to prepare qPCR reaction mixtures with GAPDH as an endogenous control to measure the expression levels of DCST1-AS1 and Notch1 mRNA.

PureLink miRNA Isolation Kit (Thermo Fisher Scientific) was used to extract miRNAs from the aforementioned tissues and cells. Expression levels of mature miR-92a-3p were measured using All-in-One\textsuperscript{TM} miRNA qRT-PCR Detection Kit (Genecopoeia) with U6 as endogenous control.

All PCR reactions were repeated 3 times and mean values were presented. Fold changes of gene expression were measured using 2\textsuperscript{−ΔΔCT} method.
Western Blot Assay
RIPA solution (GenePharma) was used to extract total protein from $10^5$ cells, followed by BCA assay to measure protein concentrations. Protein samples were incubated in boiling water for 12 hrs, followed by 10% SDS-PAGE gel electrophoresis to separate different proteins. Gel transfer to PVDF membranes was then performed, followed by blocking in PBS containing 5% non-fat milk for 2 hrs at room temperature. After that, incubation with rabbit anti-Notch1 (ab8925, Abcam) and GAPDH (ab37168, Abcam) at 4°C overnight was performed. After that, membranes were further incubated with goat anti-rabbit HRP (IgG) (ab6721; Abcam) for 2 hrs at room temperature. Signals were produced using ECL (Sigma-Aldrich) and Quantity One software was used to normalize signals.

Transwell Assays
The effects of transfections on the invasion and migration of HEC-1 cells were analyzed by Transwell assay using inserts from Corning (8-mm pore size). The upper chamber was filled with 0.1 mL cell suspension (serum-free) containing $10^4$ cells. The lower chamber was filled with 80% cell culture medium and 20% FBS. Cells were cultured under the aforementioned conditions for 12 hrs. The lower surface of membranes was then stained with 0.1% crystal violet in 20% ethanol for 15 min at room temperature. After that invading and migrating cells were observed and counted under a light microscope. It is worth noting that uncoated membranes were used in migration assay, while Matrigel-coated membranes were used in invasion assay.

Statistical Analysis
Three biological replicates were included in each experiment and mean values were used for data analysis. Differences between EC and non-tumor tissues were explored using a paired t test. Differences among multiple groups were explored by performing ANOVA (one-way) and Tukey’s test. Survival analysis was performed by dividing the 62 EC patients into high and low DCST1-AS1 level groups (n=31) with the median expression level of DCST1-AS1 in EC tissues as the cutoff value. Survival curves were plotted and compared by K-M plotter and log-rank test, respectively. Chi-squared test was used to analyze the correlations between the expression level of DCST1-AS1 and patients’ clinical data. p<0.05 was statistically significant.

Results
Upregulation of DCST1-AS1 in EC Predicted Poor Survival
The differential expression of DCST1-AS1 in EC was analyzed by measuring the expression levels of DCST1-AS1 in both EC and non-tumor tissues from the 62 EC patients. Paired t test showed that, comparing to non-tumor tissues, expression levels of DCST1-AS1 were significantly higher in EC tissues (Figure 1A, p<0.05). Survival curves for high and low DCST1-AS1 level groups were plotted and compared. Comparing to low DCST1-AS1 level group, the survival rate of patients in the high DCST1-AS1 level group was significantly lower (Figure 1B). Chi-squared test showed that expression levels of DCST1-AS1 were not significantly correlated with patients’ age, AJCC stage and tumor spread (including lymph node metastasis) (Table 1).

DCST1-AS1 and miR-92a-3p Cannot Regulate the Expression of Each Other
The potential interaction between DCST1-AS1 and miR-92a-3p was predicted by performing IntaRNA. It was observed that DCST1-AS1 and miR-92a-3p can form strong base pairing between each other (Figure 2A left, p<0.05). Dual luciferase reporter assay was performed by co-transfecting HEC-1 cells with either the combination of DCST1-AS1 vector + miR-92a-3p (miR-92a-3p group) or the combination of DCST1-AS1 vector + NC miRNA (NC group). Comparing to the NC group, relative luciferase activity was significantly lower in the miR-92a-3p group (Figure 2A right, p<0.05). HEC-1 cells were transfected with DCST1-AS1 vector or miR-92a-3p mimic to further analyze the interaction between them. Overexpression of DCST1-AS1 and miR-92a-3p was confirmed by qPCR at 24-hrs post-transfection (Figure 2B, p<0.05). Comparing to C and NC groups, DCST1-AS1 and miR-92a-3p overexpression failed to affect the expression of each other (Figure 2C).

DCST1-AS1 Overexpression Led to the Upregulation of Notch1
Notch1 is a target of miR-92a-3p. Effects of DCST1-AS1 and miR-92a-3p overexpression on the expression of Notch1 at mRNA (Figure 3A) and protein (Figure 3B) levels were analyzed by performing qPCR and Western blot, respectively. Comparing to C group, miR-92a-3p
overexpression led to the downregulated Notch1 expression (p<0.05). In contrast, DCST1-AS1 overexpression led to upregulated Notch1 expression and reduced effects of miR-92a-3p overexpression (p<0.05).

**DCST1-AS1 Overexpression Promoted the Invasion and Migration of HEC-1 Cells Through miR-92a-3p/Notch1 Axis**

The effects of DCST1-AS1, miR-92a-3p and Notch1 overexpression on the invasion (Figure 4A) and migration (Figure 4B) of HEC-1 cells were analyzed by performing Transwell assays. Comparing to C group, DCST1-AS1 and Notch1 overexpression led to increased cancer cell invasion and migration rates. Overexpression of miR-92a-3p played an opposite role and attenuated the effects of DCST1-AS1 overexpression (p<0.05). The expression patterns of DCST1-AS1, miR-92a-3p and Notch1 mRNA in all transfection groups are shown in Supplemental Figure 1A–C (p<0.05).

**Discussion**

In this study, we mainly investigated the roles of DCST1-AS1 in EC. We found that DCST1-AS1 was upregulated in EC and is correlated with the poor survival of EC patients. In addition, DCST1-AS1 overexpression in EC cells led to increased cancer cell invasion and migration rates. Overexpression of miR-92a-3p played an opposite role and attenuated the effects of DCST1-AS1 overexpression (p<0.05). The expression patterns of DCST1-AS1, miR-92a-3p and Notch1 mRNA in all transfection groups are shown in Supplemental Figure 1A–C (p<0.05).

**Table 1** Correlation Between Expression Level of DCST1-AS1 and Patients’ Clinical Data

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LncRNA DCST1-AS1 has recently been proven as an oncogenic lncRNA in liver cancer.\(^{13,14}\) In liver cancer, DCST1-AS1 is upregulated and may regulate multiple signaling pathways, such as FAIM2 and AKT/mTOR signaling pathways, to promote cancer cell proliferation, invasion and migration, and inhibit cancer cell apoptosis.\(^{13,14}\) This study is the first to report the upregulation of DCST1-AS1 in EC. In addition, DCST1-AS1 overexpression in EC cells led to the increased cell invasion and migration rates. Therefore, DCST1-AS1 is likely an oncogenic lncRNA in EC.

Although the survival of early-stage EC patients has been improved significantly during the past decades, the overall survival of advanced stage EC patients is still poor, largely owing to the lack of effective therapeutic
Figure 2 DCST1-AS1 and miR-92a-3p cannot regulate the expression of each other. The potential interaction between DCST1-AS1 and miR-92a-3p was predicted by performing IntaRNA. It was observed that DCST1-AS1 and miR-92a-3p can form strong base pairing between each other (A, left). Dual luciferase reporter assay was performed by co-transfecting HEC-1 cells with either the combination of DCST1-AS1 vector + miR-92a-3p (miR-92a-3p group) or the combination of DCST1-AS1 vector + NC miRNA (NC group). Luciferase activity was measured at 48-hrs post-transfection and was compared (A, right). HEC-1 cells were transfected with DCST1-AS1 vector or miR-92a-3p mimic to further analyze the interaction between them. Overexpression of DCST1-AS1 and miR-92a-3p was confirmed by qPCR at 24-hrs post-transfection (B). The effects of DCST1-AS1 and miR-92a-3p overexpression on the expression of each other were also analyzed by qPCR at 24-hrs post-transfection (C). Experiments were repeated 3 times and mean values were presented. *p<0.05.

Figure 3 DCST1-AS1 overexpression led to the upregulation of Notch1. Notch1 is a target of miR-92a-3p. Effects of DCST1-AS1 and miR-92a-3p overexpression on the expression of Notch1 at mRNA (A) and protein (B) levels were analyzed by performing qPCR and Western blot, respectively. Experiments were repeated 3 times and mean values were presented. *p<0.05.
approaches.\textsuperscript{16,17} Therefore, the accurate prognosis of EC may help to improve the long-term survival of EC patients by guiding the selection of therapies and the development of the postoperative care system. This study proved that the high expression level of DCST1-AS1 was closely correlated with the poor survival of EC patients. Therefore, measurement of the expression level of DCST1-AS1 in EC before therapies may help the prognosis of EC, while the accuracy remains to be further tested.

In a recent study, Song, et al reported that miR-92a-3p can target Notch1 to inhibit glioma.\textsuperscript{12} In this study, we also observed the downregulation of Notch1 in EC cells after miR-92a-3p overexpression. Therefore, miR-92a-3p may also target Notch1 in EC. In this study, we showed that DCST1-AS1 and miR-92a-3p may interact with each other, while overexpression experiments showed that DCST1-AS1 and miR-92a-3p overexpression did not significantly affect the expression of each other. Instead, DCST1-AS1 overexpression led to the upregulation of Notch1, which is the target of miR-92a-3p. Therefore, DCST1-AS1 may “absorb” or “sponge” miR-92a-3p to upregulate Notch1, which in turn promotes the invasion and migration of EC cells. However, other mechanisms may also exist. More studies are still needed.

\section*{Conclusion}

In conclusion, DCST1-AS1 is upregulated in EC and may regulate miR-92a-3p/Notch1 to promote EC cell invasion and migration.

\section*{Funding}

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\section*{Disclosure}

The authors report no conflicts of interest in this work.

\section*{References}