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Increased expression of lncRNA HULC in human epithelial ovarian cancer and its biological functions

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Summary

Purpose: Long noncoding RNA (lncRNAs) highly upregulated in liver cancer (HULC) has recently been revealed to be upregulated in several human malignancies and correlated with tumor progression. The purpose of this study was to explore the role and function of HULC in human epithelial ovarian cancer (EOC). *Materials and Methods:* LncRNA HULC expression in EOC tissues and cell lines was detected by quantitative real-time PCR. Then, the association between HULC expression and clinicopathological characteristics and patient survival was analyzed. Furthermore, the effects of HULC on the biological behavior of EOC cells were evaluated by in vitro assays. *Results:* HULC was significantly upregulated in EOC tissues and cell lines. High level of HULC was associated with advanced FIGO Stage, higher serum CA125 expression level, lymph node metastasis, and poor overall survival. Knockdown of HULC in OVCAR3 was able to reduce cell proliferation, promote cell apoptosis, and inhibit cell invasion. *Conclusions:* These findings indicate that HULC may exert oncogenic activity in EOC and would serve as a novel prognostic biomarker and therapeutic target for EOC.

Key words: LncRNA HULC; Epithelial ovarian cancer; Prognosis; Proliferation; Invasion.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy worldwide [1]. Inspite of the development of diagnostic and therapeutic technologies, approximately 70% of EOC patients are diagnosed with advanced FIGO Stages (III or IV), and the five -year survival rate is less than 40% for these patients [2, 3]. Understanding the molecular mechanisms of EOC and identifying novel biomarkers and therapeutic targets would be of great importance to the improvement of clinical outcomes of this disease.

Long noncoding RNAs (lncRNAs) are transcripts longer than 200 nucleotides with little or no protein coding function [4]. As one of the key members of gene regulatory networks, lncRNAs take part in epigenetic regulation and are involved in diverse biological processes as well as disease pathogenesis [5]. LncRNAs are dysregulated in different kinds of cancer and exert critical functions in cancer biology [6-11]. Increasing evidence has shown that aberrant expression levels of certain lncRNAs are associated with EOC initiation and progression. For example, overexpression of lncRNA ZFAS1 promoted EOC cell proliferation and migration and induced chemoresistance [12]. Increased expression of lncRNA NEAT1 in EOC tissues was correlated with advanced clinical stage and distant metastasis[13]. Upregulation of lncRNA SPRY4-IT1 indicated poor prognosis of EOC patients [14]. Plasma MALAT1 may act as a valuable biomarker for the early diagnosis of EOC [15].

A new lncRNA, highly upregulated in liver cancer (HULC), located on human chromosome 6p24.3, was originally identified as an upregulated gene in hepatocellular carcinoma (HCC) and exerted oncogenic functions [16]. Subsequent studies demonstrated increased HULC expression and its oncogenic properties in glioma [17], cholangiocarcinoma [18], gastric cancer [19], osteosarcoma [20], cervical cancer [21], and colorectal cancer [22]. However, the expression and significance of HULC in EOC has not been evaluated yet. In the present study, the authors examined the expression level of HULC in human EOC tissues and cell lines, and then explored the association between HULC expression and clinicopathological characteristics. Furthermore, they investigated the biological function of HULC in EOC cells.

Materials and Methods

A total of 95 primary EOC tissues and 45 normal ovarian tissue specimens were collected during surgery at the Department of Obstetrics and Gynecology, Provincial Hospital Affiliated to Shandong University (Jinan 250021, China) from 2008 to 2011. None of the EOC patients were treated with radiotherapy, chemotherapy or hormonal therapy prior to surgery. The normal ovarian tissues were obtained from women who underwent hysterectomies for benign disease. All tissue samples were snap-frozen in liquid ni-

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trogen and then stored at -80°C. The clinicopathological variables of patients are shown in Table 1. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Shandong University.

The human EOC cell lines (OVCAR3, Caov3, and SKOV3) and normal human ovarian surface epithelial (HOSE) cells were acquired. EOC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. HOSE cells were cultured in medium containing 1:1 mixture of MCDB 105 and M199 medium. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

For HULC knockdown, EOC cells were seeded into each well of six-well plates and incubated for 24 hours, and then transfected with siRNA oligonucleotides using Lipofectamine 2000 according to the manufacturer's protocol. The si-HULC (target sequence: 5'-GCCTTTACAAGGGAATGAAGA-3') and negative control siRNA (si-NC) were provided.

Total RNA was isolated from tissue samples and cells using TRIzol reagent according to the manufacturer's instructions. cDNA was generated by reverse transcription from 1 μg of total RNA using a PrimeScript 1st Strand cDNA synthesis kit. Real-time PCR was performed with SYBR Premix Ex Taq II. Results were normalized to an endogenous control (GAPDH) using the 2^{-DCt} method. The primers for HULC were 5'-CCA GAG GAG GAG GTA GGG AC-3' (sense) and 5'-TGA TGT GAG TCT GGG CTG AG-3' (reverse); the primers for GAPDH were 5'-CAG CCA GGA GAA ATC AAA CAG -3' (sense) and 5'-GAC TGA GTA CCT GAA CCG GC-3' (reverse).

Cell proliferation was evaluated using a cell counting kit at 0, 24, 48, 72 and 96 hours after transfection. Briefly, EOC cells were plated in 96-well plates at a concentration of 5×10^3 cells/well. Then 10 μL CCK-8 solution was added to each well and incubated for one hour. The absorbance at 450 nm was measured using a microplate reader.

The annexin V-FITC apoptosis detection kit was used to detect cell apoptosis rate. After HULC siRNA transfection, EOC cells were collected, washed with ice-cold PBS, and resuspended at a concentration of 1×10^6 cells/ml. Then, the cells were stained with 5 ml of annexin V-FITC and 10 ml of propidium iodide (PI, 20 μg/ml), and incubated in the dark at 25°C for 15 minutes. Cell apoptosis was analyzed using a FACScan flow cytometer.

Transwell assay was performed to evaluate the invasion ability of EOC cells transfected with si-HULC using Matrigel-coated cell culture chambers (8-μm pore size). Briefly, 48 hours after transfection, EOC cells were resuspended in 200 μL serum-free medium and seeded into the upper chambers. DMEM containing 20% FBS was added into the lower chamber as a chemoattractant. After 24 hours of incubation, the cells remaining on the upper membrane were carefully removed. Cells that had invaded through the membrane were fixed with methanol and stained with 0.1 % crystal violet, and counted using a microscope. Five fields were randomly chosen for each sample.

The SPSS19.0 software was used for general statistical analyses, and $p < 0.05$ was considered statistically significant. The significance of differences between groups was performed using Student's *t*-test or chi-square test. Overall survival was defined as the time interval from the date of surgery to death or to the last follow-up. Survival curves were estimated with the Kaplan–Meier method, and compared using the log-rank test. Univariate and multivariate analyses were performed using Cox proportional hazards regression models, and hazard ratios (HRs) for variables were calculated.

Table 1. — *Correlation between lncRNA HULC expression and clinicopathological characteristics of EOC patients.*

	High HULC expression (n, %)	Low HULC expression (n, %)	*p*-value
Age (years)			
≥ 50	35 (56.5%)	27 (43.5%)	
< 50	13 (39.4%)	20 (60.6%)	0.135
Histological type			
Serous	16 (55.2%)	13(44.8%)	
Mucinous	21 (47.8%)	22 (52.2%)	
Others	11 (48.8%)	12 (51.2%)	0.367
Histological grade			
G1	24 (53.3%)	21 (46.7%)	
G2	10 (47.6%)	11 (52.4%)	
G3	14 (48.3%)	15 (51.7%)	0.272
FIGO Stage			
I/II	14 (33.3%)	28 (66.7%)	
III/VI	34 (64.2%)	19 (35.8%)	0.004
Serum CA 125 level (U/L)			
$< 5.0 \times 10^5$	15(34.1%)	29 (65.9%)	
$\geq 5.0 \times 10^5$	33 (64.7%)	18 (35.3%)	0.003
Ascites			
Yes	26(53.1%)	23 (46.9%)	
No	22 (47.8%)	24 (52.2%)	0.380
Lymph node involvement			
No	18 (36.7%)	31 (63.3%)	
Yes	30 (65.2%)	16 (34.8%)	0.008

Table 2. — *Univariate and multivariate analysis of prognostic variables by Cox regression analysis.*

Variables	Univariate analysis		Multivariate analysis	
	HR	*p*-value	HR	*p*-value
Age (years)(≥ 50 / < 50)	0.95	0.581	--	--
Histological type (Serous/non-serious)	0.76	0.712	--	--
Histological grade (G1/G2+G3)	2.03	0.031	1.36	0.095
FIGO Stage (III+IV/I+II)	3.87	0.003	2.95	0.007
Ascites (yes/no)	1.18	0.132	--	--
Serum CA 125 ($\geq 5.0 \times 10^5 / < 5.0 \times 10^5$)	1.21	0.108	--	--
Lymph node involvement (yes/no)	2.94	0.009	2.27	0.011
Expression of HULC (low/high)	4.76	<0.001	3.81	0.004

Results

The authors performed quantitative RT-PCR analysis to detect HULC expression in EOC tissues and cell lines. Figure 1A shows that the expression level of HULC in EOC

tissues was significantly higher than that in normal epithelial ovarian tissues ($p < 0.001$). In addition, HULC expression was significantly increased in three EOC cell lines compared with normal HOSE cells (Figure 1B). The OVCAR3 cell line exhibited the highest HULC expression and was thus chosen for si-HULC transfection and subsequent in vitro experiments.

To assess the associations between HULC expression and clinicopathological features, the 95 EOC patients were divided into high HULC expression group and low HULC expression group, using the median value of HULC expression in all EOC tissues as a cutoff. As shown in Table 1, HULC upregulation was significantly associated with advanced FIGO Stage ($p = 0.004$), higher serum CA125 expression level ($p = 0.003$), and lymph node metastasis ($p = 0.008$). However, HULC expression was not associated with other clinicopathologic features of EOC patients, including age, ascites, histological type, as well as histological grade.

The Kaplan-Meier analysis and log-rank test were used to evaluate the relationship between HULC expression and patient survival. The authors found that high HULC expression was significantly associated with poor overall survival in EOC patients (Figure 2). Univariate Cox regression analysis revealed that HULC expression ($p < 0.001$), tumor differentiation ($p = 0.031$), lymph node metastasis ($p = 0.009$), and FIGO Stage ($p = 0.003$) were statistically significant risk factors affecting the overall survival (Table 2). Multivariate Cox regression analysis indicated that HULC expression (HR = 3.81, $p = 0.004$), lymph node status (HR = 2.27, $p = 0.011$), and clinical stage (HR = 2.95, $p = 0.007$) were independent prognostic factors for patients with EOC (Table 2).

To explore the role of HULC in EOC, OVCAR3 cells were transfected with si-HULC or si-NC. Figure 3A confirmed decreased HULC expression after si-HULC transfection. The CCK-8 assay revealed that downregulation of HULC significantly reduced the proliferation of OVCAR3 cells (Figure 3B). Next, flow cytometric analysis was performed to examine the effects of HULC on OVCAR3 cell apoptosis. As shown in Figure 3C, knockdown of HULC resulted in a significant increase in the apoptosis rate of OVCAR3 cells. Finally, the results of Transwell invasion assay indicated that the invasion capacity of OVCAR3 cells was significantly reduced after si-HULC transfection (Figure 3D).

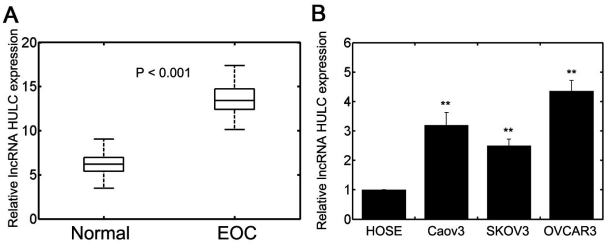

Figure 1. — Expression of lncRNA HULC in epithelial ovarian cancer (EOC) tissues and cell lines.
(A) HULC expression is significantly higher in EOC tissues than in normal ovarian tissues.
(B) HULC expression is upregulated in human EOC cell lines OVCAR3, Caov3, and SKOV3, compared to normal human ovarian surface epithelial (HOSE) cells. $^{**}p < 0.01$.

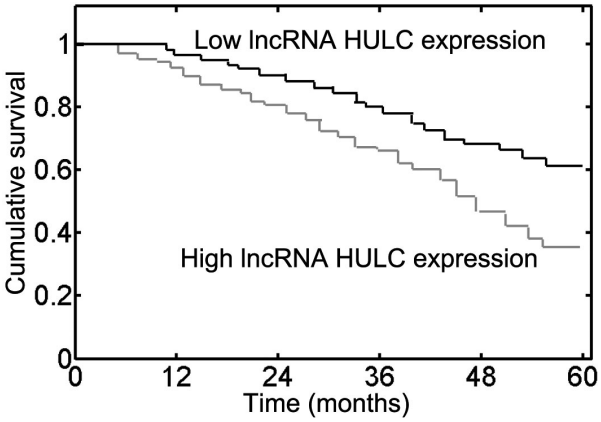

Figure 2. — Kaplan-Meier survival curves of patients with EOC based on HULC expression levels. Patients in the high HULC expression group have a significantly poorer prognosis than those in low HULC expression group ($p < 0.001$, log-rank test).

Discussion

Emerging evidence suggests that lncRNAs play a crucial role in the carcinogenesis and cancer progression [8, 23, 24]. However, limited data are available on the expression and function of lncRNAs in EOC. In the present study, the authors found the expression of HULC to be significantly upregulated in EOC specimens as compared with that in normal ovarian tissues. This increased expression of HULC was correlated with lymph node metastasis, advanced clinical stage, and poor overall survival. COX regression analysis further identified HULC overexpression as an independent predictor of poor survival of EOC patients. Furthermore, in vitro functional assays demonstrated that downregulation of HULC could efficiently inhibit EOC cell proliferation and invasion and promote cell apoptosis. To the present authors' knowledge, this is the first study to analyze the expression pattern, clinical significance, and biological function of HULC in EOC.

HULC overexpression and its oncogenic properties have been reported in several human malignancies. HULC was upregulated in HCC tissues and associated with TNM stage, intrahepatic metastases, and HCC recurrence [25]. HULC depletion inhibited HCC growth and metastasis [25]. A high serum HULC level was correlated with tumor

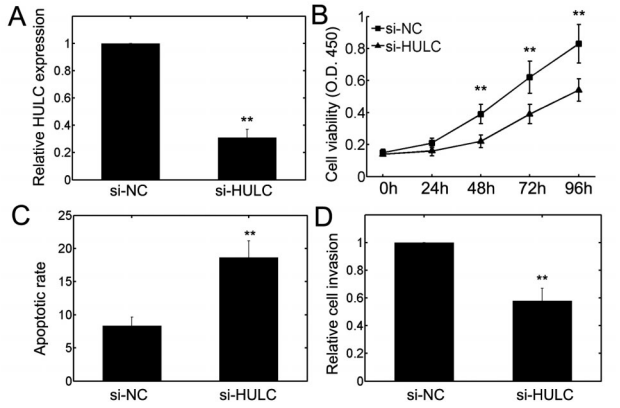

Figure 3. — Downregulation of HULC inhibits OVCAR3 cell proliferation and invasion and promotes apoptosis.
(A) HULC expression levels are evaluated using qRT-PCR in si-HULC transfected OVCAR3 cells. $^{**}p < 0.01$.
(B) CCK-8 assay is performed to determine the proliferation of OVCAR3 cells transfected with si-HULC. $^{**}p < 0.01$.
(C) Flow cytometric analysis shows that downregulation of HULC promoted OVCAR3 cell apoptosis. $^{**}p < 0.01$.
(D) Transwell invasion assay is performed in OVCAR3 cells transfected with si-HULC to investigate changes in cell invasion. $^{**}p < 0.01$.

size, lymph node metastasis, distant metastasis, clinical stage, and H. pylori infection in gastric cancer [19]. Increased HULC expression in pancreatic cancer was closely related to large tumor size, lymph node metastasis, and vascular invasion [26]. In addition, HULC overexpression predicted poor survival in patients with HCC [25], gastric cancer [19], glioma [17], osteosarcoma [20], diffuse large B-cell lymphoma [27], and pancreatic cancer [26]. Gain- and loss-of-function studies revealed that HULC could promote cell proliferation, colony formation, migration and invasion, and inhibit cell apoptosis in HCC [28], gastric cancer [29], osteosarcoma [30], glioma [31], and cholangiocarcinoma [18]. Collectively, HULC might play important role in tumor initiation and development, and would serve as a novel biomarker and potential therapeutic target in human malignancies.

In conclusion, the present study showed that lncRNA HULC expression was upregulated in EOC tissues and cells, and its overexpression was associated with aggressive clinicopathological features and poor prognosis. Knockdown of HULC obviously reduced EOC cell proliferation and invasion and promoted apoptosis in vitro. These findings demonstrated that HULC might be a potential prognostic biomarker and therapeutic target for EOC. Future study should be performed to clarify the detailed molecular mechanism by which HULC exerts oncogenic functions.

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