The *HSP70* gene predicts prognosis and response to chemotherapy in epithelial ovarian cancer

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Background: Chemotherapy resistance is an intractable problem in treating patients with epithelial ovarian cancer (EOC). Heat shock proteins (HSPs) act as apoptosis inhibitors and are highly conserved genetically. Most HSPs have strong cytoprotective effects, and their overexpression inhibits apoptosis. This has been demonstrated for *HSP70*. Heat shock protein 70 (*HSP70*) expression is abnormally upregulated in malignant cells. Furthermore, *HSP70* can inhibit cell death and promote chemotherapeutic resistance. In our study, the relationship between the *HSP70* gene and primary chemotherapy resistance and clinical outcome in patients with EOC was explored.

Methods: Quantitative real-time polymerase chain (qRT-PCR) was applied to determine *HSP70* messenger RNA (mRNA) levels, and immunohistochemistry assay was conducted to determine *HSP70* protein level. *HSP70* overexpression was assessed to clarify its role on chemotherapy resistance to cisplatin in SKOV3 cell lines.

Results: RT-qPCR assay indicated a strong relationship between *HSP70* expression and chemotherapy resistance in patients with EOC. In cultured SKOV3 cells, overexpression of *HSP70* inhibited cell sensitivity to cisplatin. Kaplan-Meier analysis demonstrated high *HSP70* expression was associated with poor outcome of EOC patients. In multivariate models, high *HSP70* expression independently predicted this poor outcome.

Conclusions: *HSP70* predicts the prognosis and response to chemotherapy in EOC patients.

Keywords: Heat shock protein 70 (*HSP70*); epithelial ovarian cancer (EOC); platinum resistance; prognosis; gene

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Introduction

Epithelial ovarian cancer (EOC) has the highest incidence and mortality among all gynecologic malignancies (1). The median time to recurrence of advanced ovarian cancer is less than 2 years (2), and the 5-year overall survival (OS) rate is about 30% (3). Presently, the standard therapies for EOC patients with advanced stage consists of surgeries and platinum-based chemotherapy (4). However, primary chemotherapy is ineffective in more than 20% patients (5). After primary chemotherapy, 70% of patients can achieve complete clinical remission; however, more than 85% of them will ultimately relapse and become resistant to chemotherapy (6). Chemotherapy resistance, either primary or acquired, is the main obstacle to successful treatment and remains a major problem in the management of patients with EOC.

Table 1 Clinical characteristics of 154 EOC patients

Characteristics	Stage	Patients (n)	Median	Percentage/range
Age	<50 years	56	58 years	36.4%
	≥50 years	98	58 years	63.6%
Histology	Serous	96		62.3%
	Endometrioid	35		22.72%
	Mucinous	9		5.84%
	Clear cell	5		3.2%
	Mixed type	9		5.84%
FIGO stage	I–II	34		22.1%
	III–IV	120		77.9%
Grade	1	39		25.3%
	2	68		44.2%
	3	47		30.5%
Tumor residual size	0	41		26.6%
	<1 cm	76		49.4%
	>1 cm	37		24.0%
Platinum-based	Cisplatin	41		26.6%
	Carboplatin	113		73.4%
Follow-up time		154	37.2 months	2–60 months

EOC, epithelial ovarian cancer.

Heat shock proteins (HSPs) act as apoptosis inhibitors and are highly conserved genetically (7). Most HSPs have strong cytoprotective effects, and their overexpression inhibits apoptosis (8,9). This has been demonstrated for *HSP70* (10,11). In addition, *HSP70* expression is abnormally high in malignant cells, and overexpression of *HSP70* is related to chemotherapy resistance and lymph node metastasis (12,13). Numerous studies have indicated that reduced expression of *HSP70* in cells may improve the effectiveness of cancer treatment (14,15). Therefore, we hypothesized that *HSP70* expression may be involved in chemotherapy resistance in patients with EOC via inhibiting ovarian cancer cell apoptosis.

In this research, the relationship between the levels of *HSP70* messenger RNA (mRNA) and protein in EOC tissue with clinical prognosis was investigated. In addition, the role of *HSP70* in the viability and apoptosis of cultured ovarian cancer cells was further examined. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/atm-21-2087).

Methods

Tissue sample collection

Between December 2012 and June 2015, tumor tissue samples from EOC patients admitted to the Department of Gynecology in the Fourth Hospital of Hebei Medical University, China, were collected. The detailed clinical pathological information of EOC patients are shown in *Table 1*. All patients were fully informed of the study and signed informed consent. The study was approved by the Institutional Medical Ethics Committee of the Fourth Hospital of Hebei Medical University (No. 2017ME96). This study was performed according to the Declaration of Helsinki (as revised in 2013).

Based on their platinum-free interval (PFI), the patients were separated into a chemotherapy-resistant group (n=64) and a chemotherapy-sensitive group (n=90). A PFI of <6 months was considered to indicate chemotherapy resistance, whereas a PFI of >6 months was considered to indicate chemotherapy sensitivity (16). The participants were followed up regularly

for more than 5 years. Progression-free survival (PFS) and OS were used to analyze the survival status of EOC patients.

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

TRIzol reagent (Generay Biotech, Shanghai, Co., Ltd., China) was obtained to extract total RNA of tissue and cell samples. Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was applied to synthesize complement DNA (cDNA). For qRT-PCR assay, the primers of *HSP70* and *GAPDH* were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The internal reference was set as *GAPDH*. QuantiNova TMSYBR® Green PCR Kit (Qiagen, Hilden, Germany) was used to conduct qRT-PCR reactions in a Mx3005P instrument. All detection experiments were conducted 3 times. The $2^{-\Delta Ct}$ method was used to determine the relative expression of mRNA.

HSP70 immunohistochemistry study of clinical samples

EOC tissue samples were embedded in paraffin in the pathology department of the Fourth Hospital of Hebei Medical University. The expression of *HSP70* was evaluated with immunohistochemical (IHC) staining. Rabbit antihuman *HSP70* (SA0379, 1:1,000 dilution; Hangzhou HuaAn Biotechnology Co., Ltd., China) was applied to detect *HSP70*. Immunoreactivity for *HSP70* was considered positive in tumor cells showing cytoplasmic staining without nuclear staining. Positive staining area and intensity reflected *HSP70* expression level. IHC scores <4 indicated low *HSP70* expression, while IHC ≥4 indicated high *HSP70* expression (17).

Cell culture

To detect the role of *HSP70* on EOC cells, SKOV3 cell lines were purchased from Icell Bioscience Inc., (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (FBS; Invitrogen Gibco, NY, USA) was applied to culture SKOV3 cells. The cells were maintained in an incubator (37 ℃, humidified, constant humidity, 5% CO_2). All detections were conducted 3 times.

Transient transfection

The pCMV6-HSP70 vector and empty vector (OriGene Technology, Beijing, China) were transfected into SKOV3 cells with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. At indicated time points, the cells were collected, and the expression of *HSP70* mRNA was detected by qRT-PCR. Thus, the transfection efficacy could be determined.

Cell viability detection

The cell viability of cultured SKOV3 was analyzed by Cell Counting Kit-8 (CCK-8) assay. These cells were seeded in 96-well-plates. The seeding cell density was set at 15% confluence. The cells were cultured in fresh DMEM medium mixed with 10% FBS, penicillin, and streptomycin. At the end of cell viability assay, the cultured cells were in the growth phase. The day after seeding, different concentrations of cisplatin were applied to treat cultured SKOV3 cells; 24 hours after treatment, CCK-8 was used to detect the cell viability of cultured SKOV3 cells. In this process, 10 μL of CCK-8 was added to each well; 3 hours after adding CCK-8, the absorbance of each well at 492 nM was detected by a microplate reader. Each detection was conducted in triplicate.

Cell apoptosis detection

The SKOV3 cells at the logarithmic growth phase were seeded into 6-well plates at a density of 3×10^5/mL and 2 mL per well. The cultured cells were randomly separated into the control group and experimental group. Next, 48 hours after transfection, cisplatin was applied to treat cultured cells for 24 hours. Cold phosphate-buffered saline (PBS) was applied 3 times to wash cultured cells, and the rinsed cells were collected for flow cytometry assay. This was followed by 10 minutes of double staining with Annexin V-PE in a dark room. The percentage of apoptotic cells was detected by flow cytometry assay.

Statistical analysis

The collected data in this study were analyzed with SPSS 21.0 (IBM Corp., Armonk, NY, USA). A P value <0.05 was considered statistically significant. Wilcoxon rank-sum test was applied to analyze the difference of *HSP70* mRNA expression between the 2 groups. The difference in HSP70 protein expression between the groups was analyzed with χ^2 test. The relationship between *HSP70* mRNA expression and the prognosis of EOC patients was analyzed with

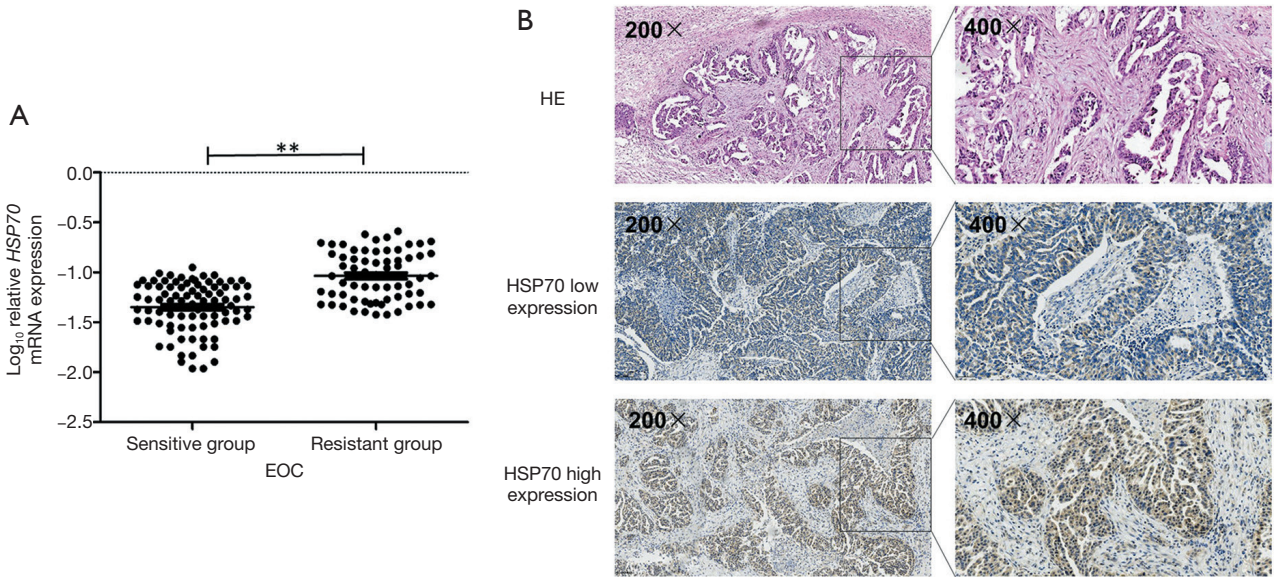

Figure 1 Expression of *HSP70* in patients with EOC. (A) *HSP70* mRNA expression was higher in chemotherapy-resistant patients compared with chemotherapy-sensitive patients. (B) Representative IHC staining of *HSP70* expression in EOC tissues. HE: representative hematoxylin and eosin staining in EOC tissues. **P<0.01. *HSP70*, heat shock protein; EOC, epithelial ovarian cancer; mRNA, messenger RNA.

Table 2 *HSP70* protein expression differences between the platinum-resistant group and the platinum-sensitive group

HSP70 expression	Resistant group n (%)	Sensitive group n (%)	P value
High	28 (77.55)	27 (56.75)	0.02
Low	8 (22.45)	22 (43.24)	

HSP70, heat shock protein.

Kaplan-Meier analysis and the Cox proportional hazards model. *t* test was applied to compare the data from *in vitro* experiments.

Results

Association between HSP70 expression and chemotherapy resistance of EOC patients

The results of qRT-PCR confirmed *HSP70* mRNA expression was significantly higher in chemotherapy-resistant patients than in chemotherapy-sensitive patients (P=0.01, *Figure 1A*). IHC assay showed *HSP70* was mostly located in the cytoplasm of EOC tumor tissue (*Figure 1B*). Positive expression of *HSP70* occurred significantly more often in the 36 chemotherapy-resistant patients than in the 50 chemotherapy-sensitive patients (P=0.02, *Table 2*).

Correlation of high expression of HSP70 with prognosis in patients with serous ovarian cancer

According to the median value of *HSP70* mRNA expression, the patients were separated into a low-expression group and a high-expression group. Kaplan-Meier analysis showed that high *HSP70* expression was associated with significantly lower PFS and OS compared as compared to low *HSP70* expression (P=0.003, *Figure 2A*; P=0.014, *Figure 2B*). After adjusting for other prognostic factors (age, stage, grade, and tumor residual size), high *HSP70* expression was also significantly associated with shorter PFS and OS (P=0.014, P=0.023; *Table 3*), demonstrating that *HSP70* expression is an independent predictor of poorer clinical outcome in patients with EOC.

HSP70 overexpression in ovarian cancer cells by pCMV6-HSP70 vector

The overexpression efficiency of pCMV6-HSP70 was detected by qRT-PCR. The expression of *HSP70* mRNA was significantly higher in the pCMV6-HSP70 group than the empty vector transfection group (P<0.001; *Figure 3A*).

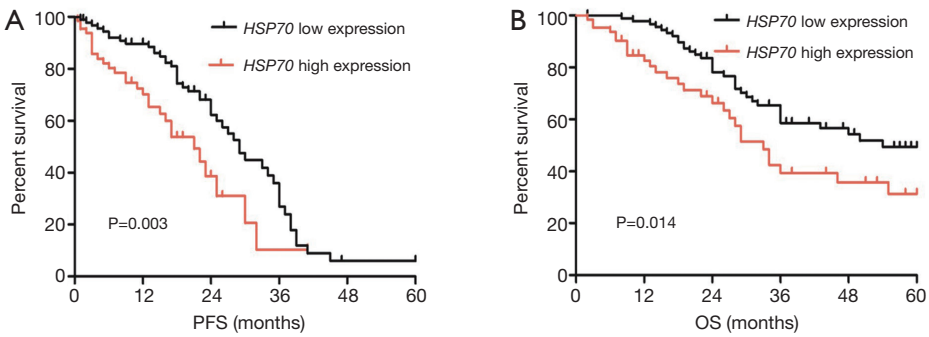

Figure 2 The association between high expression of *HSP70* and survival in 154 EOC patients. (A) Kaplan-Meier analysis of PFS according to *HSP70* mRNA expression. (B) Kaplan-Meier analysis of OS according to *HSP70* mRNA expression. *HSP70*, heat shock protein; EOC, epithelial ovarian cancer; PFS, progression-free survival; OS, overall survival.

Table 3 The association between clinical characteristics and treatment outcome in EOC patients treated with platinum-based chemotherapy

Characteristics	Recurrence		P value	Survival		P value
	HR	95% CI[a]		HR	95% CI[a]	
Age						
<50 *vs.* ≥50 years	1.06	0.64–1.77	0.820	1.23	0.73–2.10	0.440
FIGO stage						
I–II *vs.* III–IV	9.99	1.93–35.05	0.024	9.66	1.73–40.05	0.026
Grade						
G1–2 *vs.* G3	5.29	1.73–17.05	0.013	7.40	0.20–14.80	0.019
Tumor residual size						
0 *vs.* ≤1 cm	4.39	1.75–10.93	0.011	5.40	0.20–11.80	0.017
0 *vs.* >1 cm	3.09	1.05–7.93	<0.01	3.40	1.20–6.80	<0.01
HSP70 expression						
Low *vs.* high	1.48	1.27–2.33	0.014	1.62	1.31–2.31	0.023

[a], adjusted for age, stage, grade, tumor residue, and *MGRN1* expression. EOC, epithelial ovarian cancer.

Effect of HSP70 overexpression on cellular response to cisplatin

As confirmed by CCK-8 assays, SKOV3 cell viability in the pCMV6-HSP70 transfection group was evidently higher than that in empty vector transfection group at different concentrations of the cisplatin condition (P<0.05; *Figure 3B*). This indicates that upregulation of *HSP70* enhanced SKOV3 cell viability. In addition, flow cytometry confirmed that there were fewer apoptotic cells in the pCMV6-HSP70 transfection group compared with the empty vector transfection group in the 10 μM cisplatin treatment condition (P=0.025; *Figure 3C,D*).

Discussion

Our results confirm that, in patients with EOC, the expression of *HSP70* is significantly higher in chemotherapy-resistant tissue compared with chemotherapy-sensitive tissue. Moreover, higher expression of *HSP70* indicates poor clinical outcome in EOC patients, and overexpression of *HSP70* may desensitize SKOV3 ovarian cancer cells to cisplatin.

Recent evidence has indicated that high expression of *HSP70* may be associated with metastasis, poor prognosis, and resistance to chemotherapy (18,19). However, this study was first to report the association between HSP70 expression and chemotherapy resistance in EOC patients.

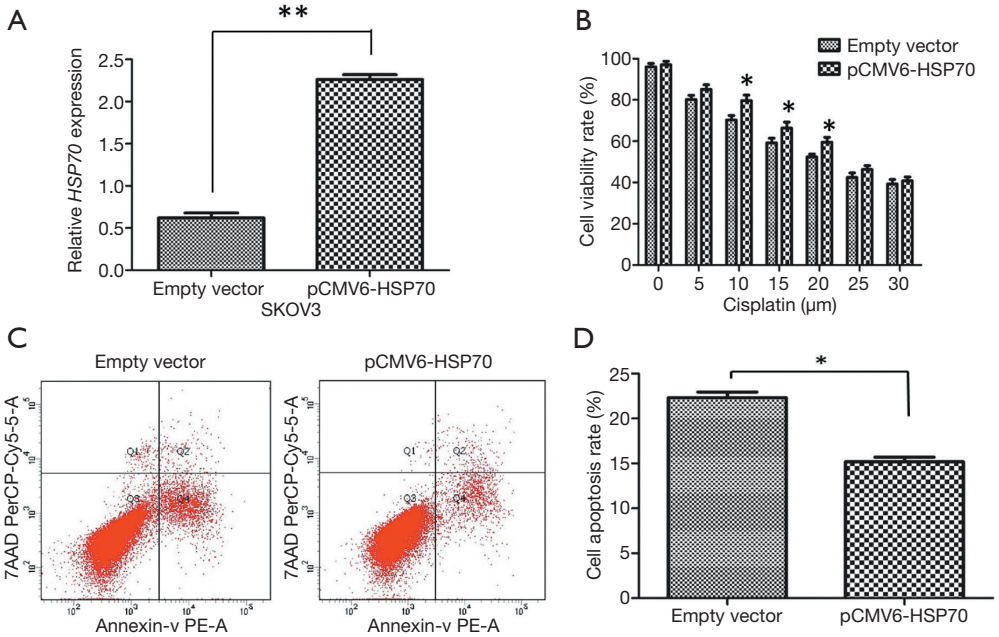

Figure 3 *HSP70* overexpression enhanced the sensitivity to cisplatin in SKOV3 cells. (A) The expression of *HSP70* in the pCMV6-HSP70 transfection group and the empty vector transfection group as detected by qRT-PCR. (B) The proliferative ability of SKOV3 cells in the pCMV6-HSP70 transfection group and empty vector transfection group detected by CCK-8 assays. (C,D) The apoptotic rate of SKOV3 cells in the pCMV6-HSP70 transfection group and empty vector transfection group detected by flow cytometry. *P<0.05; **P<0.01. *HSP70*, heat shock protein; qRT-PCR, quantitative real-time polymerase chain; CCK-8, Cell Counting Kit-8.

Our results show that mRNA levels and protein expression of *HSP70* are significantly higher in chemotherapy-resistant tissue than in chemotherapy-sensitive tissue. These results provide strong evidence that high expression of *HSP70* may have a critical role in the chemotherapy resistance in EOC. Furthermore, patients with high expression of *HSP70* had a poorer prognosis than those with low expression. Thus, high expression of *HSP70* may significantly increase patients' drug resistance to first-line platinum chemotherapy, resulting in a poorer prognosis. We also demonstrated, for the first time, that high expression of *HSP70* mRNA is associated with a poor survival rate in EOC patients. These results provide strong evidence that high expression of *HSP70* could regulate chemotherapy resistance in EOC.

HSP70 is a cell-protective protein that enhances cells, allowing them to survive in lethal conditions, and is highly conserved in evolution (10,11,20). Many models have shown that overexpression of *HSP70* inhibits apoptosis following a variety of cellular stresses, including hyperthermia, oxidative stress, or cytotoxic drugs (21-23). There is evidence showing that the overexpression of *HSP70* can induce cisplatin resistance in human ovarian cancer cells by blocking Bax mitochondrial translocation (24). Therefore, we speculated that upregulation of *HSP70* in ovarian cancer cells may suppress apoptosis by inhibiting the Bax signaling pathway, thus facilitating chemotherapy resistance. To further confirm the effect of *HSP70* on chemotherapy resistance in EOC, we investigated *HSP70* expression in SKOV3 cells. As confirmed by flow cytometry analysis, the percentage of apoptotic SKOV3 cells decreased 1.3-fold after pCMV6-HSP70 transfection. Furthermore, the viability of ovarian cancer cells increased after pCMV6-HSP70 transfection. These data suggest *HSP70* can inhibit the apoptosis of ovarian cancer cells.

In summary, our study suggests that high expression of *HSP70* could induce chemotherapy resistance through inhibiting ovarian cancer cell apoptosis. However, further studies are necessary to confirm our findings.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-21-2087). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Institutional Medical Ethics Committee of the Fourth Hospital of Hebei Medical University (No. 2017ME96). This study was performed according to the Declaration of Helsinki (as revised in 2013). All patients were fully informed of the study and signed informed consent.

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