Targeting HSF1 leads to an antitumor effect in human epithelial ovarian cancer

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Abstract. Late diagnosis and lack of specific therapeutic targets contribute to the low survival rate of patients with epithelial ovarian cancer (EOC), the most lethal gynecologic malignancy. Therefore, the screening of diagnostic markers and the identification of therapeutic targets are urgently required. Heat shock factor 1 (HSF1) has been demonstrated to be overexpressed in certain malignancies and to be involved in tumor initiation, development, transformation and metastasis. It is believed that HSF1 is a promising candidate for antitumor therapy. However, its expression pattern and function in ovarian cancer are far from being fully elucidated. Therefore, we examined the HSF1 expression in human EOC tissues, and evaluated its carcinogenesis-promoting activity in a xenograft tumor model. Examination of HSF1 expression in human EOC tissues was performed by immunohistochemical assay using ovarian tissue blots. Specific short hairpin RNA (shRNA) against HSF1 was employed to knockdown HSF1 in SKOV3 cells. Cell proliferative activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay; cell cycle distribution and apoptosis were determined by flow cytometric analysis. In normal ovarian tissues, HSF1 was barely detected, whereas, high expression of HSF1 was found in malignant EOC tissues, including serous, mucinous, endometrioid, and clear cell EOC tissues. Suppressed proliferative activity and intensified apoptosis were observed in HSF1-knockdown SKOV3 cells. In nude mouse xenografts, downregulation of HSF1 was found to cause reduced carcinogenesis, indicating the antitumor effect induced by modulation of HSF1 against EOC. Our findings suggest that HSF1 may be considered as a potential candidate diagnostic marker of human EOC, and that modulation of HSF1 could be a promising therapeutic strategy against human EOC.

Introduction

Despite improvement in the scheduling and administration of chemotherapy against human epithelial ovarian cancer (EOC), EOC remains the most lethal gynecologic malignancy, and the 5-year overall survival has achieved only a marginal improvement during the last decade (1,2). EOC is the fifth leading cause of cancer-related death in women, at least partially due to late diagnosis and frequent chemotherapy resistance. The majority of EOC patients are diagnosed with locally advanced or metastatic disease (FIGO stages III and IV) (1-3). Although initial therapy with surgery and chemotherapy may be effective, the majority of patients suffer relapse, often resulting in drug resistance. Thus, identifying diagnostic markers and developing novel therapeutic strategies are important challenges to combat EOC.

In our previous study, we successfully inhibited the proliferation of EOC cells in vitro and in a xenograft model, using a novel triazole nucleoside analog, which led to suppressed expression of heat shock factor 1 (HSF1) (4). HSF1 is the crucial transcription factor of molecular chaperones and heat shock proteins, which is a highly conserved mechanism to protect organisms against various stresses. The cytoprotective and anti-apoptotic activities of HSF1 have been well documented, and research concerning its physiological function has mainly focused on its regulatory roles in the expression of chaperone genes (5,6). Nevertheless, in the past few years, the capacity of HSF1 to orchestrate a genome-wide transcriptional program has attracted growing interest, and HSF1 has revealed an important role in multiple physiological processes (7-9).

In both human cancer cell lines and tumor tissues of various origins, including breast, colon, lung, cervix, pancreatic, prostate and mesenchymal tumors, highly amplified HSF1 was detected, while low expression of the protein was observed in normal cells (9-15). There is a growing understanding of the carcinogenesis-promoting role of HSF1, and its overexpression has been found to be associated with reduced survival (7,8,11,16-19). More importantly, HSF1 orchestrates a genomic transcriptional program to support malignant transformation. Cancer cells show a large dependency on HSF1
to maintain proliferation and survival compared with normal cells (7,9,19). Additionally, HSF1 was found to regulate more genes in highly malignant cancer cells than in less aggressive or non-transformed cells, and many of these genes are cancer-specific (7). Therefore, HSF1 is regarded as an attractive target for cancer therapy (16,20-23). Despite the well-uncovered physiological roles of HSF1 in diverse tumor types, its expression pattern and carcinogenesis function in EOC remain elusive.

Recently, we reported a nucleoside analog that attenuated HSF1 expression (4). The finding motivated us to define the role of HSF1 in ovarian carcinogenesis and to address the correlation between inhibition of the HSF1 expression and the antitumor effect in EOC. The objective of present study included the investigation of the HSF1 expression pattern in EOC tissues and the antitumor effect of HSF1 in EOC cells.

Materials and methods

Tissue collection and immunohistochemical assay. Healthy and EOC ovarian tissue biopsies were purchased from Alenabio (Xi’an, China) and Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). The collected tissues included 42 benign (30 serous cystadenoma, 12 mucinous cystadenoma), 9 borderline (7 serous adenocarcinoma, 2 mucinous adenocarcinoma), 126 malignant (88 serous adenocarcinoma, 11 mucinous adenocarcinoma, 17 endometrioid adenocarcinoma, 10 clear cell carcinoma) and 62 healthy ovarian tissues. The stages and histological grades were established according to the FIGO criteria.

A rabbit polyclonal antibody (4356; Cell Signaling Technology, Inc., Danvers, MA, USA) was purchased from Alenabio and Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). The collected tissues included 42 benign (30 serous cystadenoma, 12 mucinous cystadenoma), 9 borderline (7 serous adenocarcinoma, 2 mucinous adenocarcinoma), 126 malignant (88 serous adenocarcinoma, 11 mucinous adenocarcinoma, 17 endometrioid adenocarcinoma, 10 clear cell carcinoma) and 62 healthy ovarian tissues. The stages and histological grades were established according to the FIGO criteria.

Cell proliferation assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay was employed to assess the cell viability and to determine cell proliferative activity. The assay was performed according to a previously published procedure (4). Briefly, ~1x10^5 cells were seeded per well in a 96-well plate, and incubated in 100 µl medium. Fresh medium was added every 48 h. For cell viability assessment, 10 µl MTT (5 mg/ml) was added, followed by a 4-h incubation at 37°C. The medium was replaced by 150 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich), and absorbance at 490 nm was determined in a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each assay contained at least six wells and all experiments were independently repeated at least 3 times.

Flow cytometric analysis. To determine the cell cycle distribution, cells in the logarithmic phase were dissociated by 0.25% trypsin-ethylenediaminetetraacetic acid (Life Technologies, Carlsbad, CA, USA), seeded in a 6-well plate and then allowed to attach and proliferate for 24 h. Cells were harvested by trypsin digestion, rinsed with phosphate-buffered saline (PBS), and fixed with 70% cold-ethanol for at least 12 h at -20°C. The resultant cells were centrifuged and resuspended, and were treated with RNase A for 30 min at 37°C. Then propidium iodide (PI) was added and incubated at 4°C for 30 min.

Annexin V-PE/7-AAD Apoptosis Detection kit (KeyGen, Nanjing, China) was used to evaluate cell apoptosis. Cells in a logarithmic phase were dissociated and seeded in 6-cm dish. After a 48-h culture, the resulting cells were harvested by trypsin digestion, and analyzed by following the manufacturer's instructions.

The flow cytometric detection and data analysis were performed with a flow cytometer (BD Accuri™ C6; BD Biosciences, Franklin Lakes, NJ, USA).

RNA extraction and quantitative PCR. RNA preparation and quantitative PCR were performed as described in the literature (4,24). Cells were harvested and rinsed, and RNA was extracted with TRizol reagent (Life Technologies) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with DNase I (Fermentas, Hanover, MD, USA). Reverse-transcription was performed with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Quantitative PCR was performed with the SYBR Real-Time PCR kit (GenePharma) on a Mastercycler ep realplex (Eppendorf, Hamburg, Germany).

DNA oligo pairs used in this study were as follows: GAPDH forward, GAGTCAACGGATTTGGTCGT; GAPDH reverse, TTG ATTTTGGAGGATCTCG; HSF1 forward, CATGAAAGCAT GAAATGGGCT and reverse, ACTGCACCAGTGAGATCAGAGA. Relative RNA concentration was calculated from cycle thresholds and GAPDH was used as the internal standard control. Three independent experiments were performed.
Immunoblot assay. Routine western blot analysis was carried out according to a previously published protocol (4). Anti-human HSF1 rabbit polyclonal antibody (4356; Cell Signaling Technology, Inc.) and anti-human β-actin mouse monoclonal antibody (A5441; Sigma-Aldrich) were used as primary antibodies. Horseradish peroxidase (HRP)-conjugated antibodies (goat anti-rabbit IgG-HRP, 111-035-003; goat anti-mouse IgG-HRP, 115-035-003; Jackson ImmunoResearch Inc., West Grove, PA, USA) were applied as secondary antibodies. Immunoblot signals were visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

Xenograft tumor construction. Animal maintenance and experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee of Shanghai Jiaotong University (Shanghai, China). SKOV3-NC and SKOV3-shHSF1 cells in a logarithmic phase were harvested by trypsin digestion and washed twice with PBS. Cells (1x10^6) were injected subcutaneously into 4-week-old female BALB/c nude mice. After 2 weeks, the tumor growth of SKOV3-NC cells was well-established. The mouse weight and tumor size were monitored every 3 or 4 days. At the end of the experiment, the mice were sacrificed by cervical dislocation, and tumors were excised.

Results

**HSF1 expression is elevated in malignant EOC tissues.** Hyperexpression of HSF1 is considered to be an aggressiveness marker in certain malignant carcinomas. However, to the best of our knowledge the clinical and prognostic significance of HSF1 expression in ovarian neoplasms has not been well studied. In order to investigate whether HSF1 is elevated in EOC patients, the HSF1 expression pattern was determined in normal ovarian tissues and EOC tissue samples, which were derived from patients.

We investigated the relationship of HSF1 in EOC to clinical parameters in a series of primary tumors. Forty-two benign (30 serous cystadenoma, 12 mucinous cystadenoma), 9 borderline (7 serous adenocarcinoma, 2 mucinous adenocarcinoma), 126 malignant (88 serous adenocarcinoma, 11 mucinous adenocarcinoma, 17 endometrioid adenocarcinoma, 10 clear cell carcinoma) and 62 healthy ovarian tissues were included. The expression of HSF1 was examined by immunohistochemical staining. As shown in Fig. 1A, HSF1 was barely detected in the normal ovarian tissues. The expression of HSF1 was low and largely restricted in the infiltrating stroma and tumor areas bordering necrosis in the benign and border-
HLF1 knockdown leads to reduced growth and induced apoptosis in SKOV3 cells. As discussed above, HSF1 represents an attractive therapeutic target for several human cancers, and our data confirmed its elevated expression in EOC. Thus, we aimed to ascertain whether targeting HSF1 leads to antitumor activities in EOC. To demonstrate the potential effect of targeting HSF1 in EOC treatment, we firstly investigated the biological function of HSF1 knockdown in SKOV3 cells, which is an EOC-derived immortalized cell line.

In the attempt to obtain stable HSF1-knockdown SKOV3 cells, scramble and HSF1-specific shRNA lentiviral particles were generated and transfected into SKOV3 cells, generating SKOV3-NC and SKOV3-shHSF1 cell lines respectively. Both mRNA and protein expression levels of HSF1 were examined to confirm the silencing efficiency. As expected, the expression of HSF1 was silenced in the SKOV3-shHSF1 cells, while its expression remained the same in the SKOV3-NC cells (Fig. 3A and B).

To assess the significance of targeting HSF1 in cell proliferation regulation, cell viability was determined by MTT assay, and a growth curve was evaluated for the SKOV3-NC and SKOV3-shHSF1 cells. Notably, a reduction in proliferation was observed in the SKOV3-shHSF1 cells (Fig. 3C), indicating that downregulation of HSF1 led to inhibition of cell growth.

Cell cycle distribution and cell apoptosis were investigated in the SKOV3-shHSF1 and SKOV3-NC cells. The percentage of early apoptotic cells was markedly increased in the SKOV3-shHSF1 cells (11.87%), relative to that in the SKOV3-NC cells (6.47%) (Fig. 4A and B). Additionally, the percentage of late apoptotic cells was slightly increased in the SKOV3-shHSF1 group (Fig. 4A). This implies that downregulation of HSF1 in the SKOV3 cells led to induction of cell apoptosis.

Then, cell cycle distribution was investigated in both SKOV3-shHSF1 and SKOV3-NC cells. As illustrated in

Table I. The HSF1 expression patterns in EOC tissues.

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>No HSF1 n (%)</th>
<th>Low HSF1 n (%)</th>
<th>High HSF1 n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal ovarian tissue</td>
<td>60 (96.77)</td>
<td>2 (3.23)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Serous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign serous cystadenoma</td>
<td>9 (30.00)</td>
<td>21 (70.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Borderline serous adenocarcinoma</td>
<td>3 (42.86)</td>
<td>4 (57.14)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Malignant serous adenocarcinoma</td>
<td>29 (32.95)</td>
<td>42 (47.73)</td>
<td>17 (19.32)</td>
</tr>
<tr>
<td>Mucinous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign mucinous cystadenoma</td>
<td>11 (91.67)</td>
<td>1 (8.33)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Borderline mucinous adenocarcinoma</td>
<td>1 (5.00)</td>
<td>1 (50.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Malignant mucinous adenocarcinoma</td>
<td>4 (36.36)</td>
<td>6 (54.55)</td>
<td>1 (9.09)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant endometrioid adenocarcinoma</td>
<td>12 (70.59)</td>
<td>3 (17.65)</td>
<td>2 (11.76)</td>
</tr>
<tr>
<td>Clear cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant clear cell carcinoma</td>
<td>1 (10.00)</td>
<td>4 (40.00)</td>
<td>5 (50.00)</td>
</tr>
</tbody>
</table>

p-values were calculated by Mann-Whitney U test. *p<0.001 compared with normal ovarian tissue; †p<0.01 compared with normal ovarian tissue; ‡p<0.05 compared with benign mucinous cystadenoma tissue. HSF1, heat shock factor 1; EOC, epithelial ovarian cancer.
Figure 2. Relationship between heat shock factor 1 (HSF1) expression and clinical features of the epithelial ovarian cancer (EOC) cases. HSF1 expression was evaluated with a semi-quantification scoring system considering both the positive percentage and staining intensity. Bars, median values. Statistical analyses were performed by Mann-Whitney U test, and statistical significance is indicated (*p<0.05, **p<0.01, ***p<0.001).

Figure 3. Characterization of SKOV3 cells with stable suppression of heat shock factor 1 (HSF1). (A) Quantitative PCR was employed to examine the transcription level of HSF1. GAPDH was used as the internal standard. The relative transcript concentration was normalized to original SKOV3 cells. Results are the average of three independent experiments, and bars indicate the standard deviations (SDs) (**p<0.001). p-values were calculated by t-test. (B) Western blot analysis was used to evaluate the protein expression of HSF1 in HSF1 stable knockdown SKOV3 cells (SKOV3-shHSF1), scramble shRNA stably transfected SKOV3 cells (SKOV3-NC) and parental SKOV3 cells (SKOV3). β-actin was employed as the loading reference. (C) Cell proliferation of SKOV3-NC and SKOV3-shHSF1 cells was examined by MTT assay. Cells (1x10⁴) in a logarithmic phase were seeded in fresh culture medium and allowed to grow for 24, 48, 72 or 96 h. Each experiment was repeated in triplicate, and error bars indicate the SDs (**p<0.01, ***p<0.001).
Fig. 4C, the percentage of cells at the G1 phase was increased in the SKOV3-shHSF1 cells, while the percentages of S and G2/M phase cells were decreased in the SKOV3-shHSF1 cells. The observed differences in cell cycle distribution confirmed that the suppression of proliferative activity resulted from HSF1 knockdown. These results demonstrated the antitumor effect of targeting HSF1 against EOC in vitro.

Depletion of HSF1 in SKOV3 cells results in reduced tendency of carcinogenesis. An animal study was performed to confirm the antitumor activity arising from the downregulation of HSF1 in vivo. After injection into immunodeficiency nude female mice for 14 days, the control cells formed noticeable tumors. However, the HSF1-depleted cells (SKOV3-shHSF1 cells) exhibited no tumor genesis until 39 days post-injection (Fig. 5A). In addition, the body weights of the mice were also recorded, and no discernible weight alteration was observed between the two groups (Fig. 5B). These data revealed that targeting HSF1 in SKOV3 cells also led to an antitumor effect in vivo, suggesting that HSF1 may be used as a potential therapeutic target against EOC.

Discussion

The biological functions of HSF1 in promoting cell protection and survival against stress have been well understood for decades. In addition, it is believed that the regulatory function of HSF1 is performed by coordinating chaperone protein expression. However, in the past few years, the biological function of HSF1 has been found to be more extensive than previously assumed, especially in regards to tumor cells (7,9,11). Increasing evidence indicates this ancient transcriptional regulator to be a genome-wide regulator of carcinogenesis and tumor progression. The critical cellular roles of HSF1 include maintaining or promoting tumorigenesis, facilitating tumorigenesis and malignant transformation, maintaining protein homostasis and hindering cell apoptosis. More importantly, several lines of evidence suggest that downregulation of HSF1 is a promising antitumor strategy for several types of malignancies. Therefore, HSF1 has been regarded as a promising candidate target for cancer therapy (20-23), and strategies that are capable of modulating HSF1 expression to elicit anticancer activity have recently raised considerable interest (25). However, data concerning the expression pattern of HSF1 in human ovarian cancer,
and the antitumor effect of HSF1 modulation against human ovarian cancer have not been investigated.

Our previous study demonstrated that suppression of HSF1 expression, probed by a nucleoside analog, efficiently inhibited the proliferation activity and tumorigenesis in EOC-derived cells (4), suggesting the potential to combat EOC by downregulation of HSF1 expression. In the present study, we evaluated the HSF1 expression pattern in human EOC tissues, and investigated the antitumor function of the attenuated expression of HSF1 in EOC-derived cell lines (SKOV3). We observed that HSF1 was highly expressed in malignant serous, mucinous, endometrioid, and clear cell ovarian carcinoma and low HSF1 expression was detected in benign serous and mucinous cystadenoma. In contrast, HSF1 was barely detected in healthy ovarian tissues.

The observed overexpression of HSF1 in human EOC tissues led us to suggest HSF1 as a molecular target against ovarian cancer. Additionally, the present study showed that HSF1 knockdown induced early apoptosis and inhibited proliferative activity in SKOV3 cells. Moreover, downregulation of HSF1 was found to result in suppression of EOC carcinogenesis.

Taken together, our results demonstrated that HSF1 expression plays an important role in the malignant potential of human EOC and HSF1 may be a promising diagnostic biomarker for malignant epithelial ovarian tumors. Furthermore, our data showed the potential value of manipulating HSF1 as a therapeutic strategy against EOC. In conclusion, our findings, although still preliminary, provided critical information concerning the understanding of the cellular function of HSF1 in ovarian cancer. This study may facilitate the early diagnosis and efficacious treatment of human EOC.

Acknowledgements

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