High HOXA9 gene expression predicts response to chemotherapy and prognosis of high-grade serous ovarian cancer patients

Xiao-fei Li¹, Hai-Bo Zhang¹ and Yan Huo²

Abstract

Objective: High-grade serous ovarian cancer (HGSOC) is a deadly malignancy. Homeobox protein A9 (HOXA9) is linked with serous papillary histotype differentiation, and inappropriate HOXA9 expression is a step in ovarian cancer that induces aberrant differentiation. This study aimed to reveal the significance of HOXA9 in HGSOC.

Methods: HOXA9 mRNA and protein expression were examined by quantitative PCR and immunohistochemistry, respectively. The chi-square test was used to evaluate associations between HOXA9 expression and clinical characteristics. The prognostic value of HOXA9 was calculated by the Kaplan–Meier method. The Kaplan–Meier Plotter database was used to assess the prognostic value of HOXA9.

Results: The mRNA and protein expression of HOXA9 were significantly upregulated in chemotherapy-resistant HGSOC compared with chemotherapy-sensitive HGSOC. The chi-square test showed that high HOXA9 expression was significantly related with grade, clinical stage, and residual disease. High HOXA9 expression was significantly associated with poor prognosis. The Kaplan–Meier Plotter database further confirmed these results. Cox hazard regression showed that high HOXA9 expression was an independent prognostic factor for survival in HGSOC patients.

Conclusion: This study showed that HOXA9 expression was associated with chemotherapy resistance and poor outcomes in HGSOC patients. High HOXA9 expression might be a prognostic indicator for HGSOC.

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Introduction

Epithelial ovarian cancer (EOC) is the deadliest malignant disease in women. Approximately 70% to 80% of deaths from EOC are associated with high-grade serous ovarian cancer (HGSOC), and overall mortality has not declined over the past decade. Most HGSOC patients are diagnosed at advanced stages due to a lack of specific symptoms, which is responsible for the poor prognosis of HGSOC including the low 5-year survival rate of 40%. Significant improvements have been made over the past three decades in surgical debulking, chemotherapy, and targeted therapy, but most HGSOC patients will ultimately relapse and become resistant to chemotherapy. It has been reported that traditional cytoreduction and platinum-based chemotherapy are effective in 80% of EOC patients. Nevertheless, the majority of patients will develop disease recurrence and eventually platinum resistance. Therefore, investigating biomarkers is important to improving chemosensitivity and could potentially improve patient survival.

The homeobox protein \textit{HOXA9} is linked with serous papillary histotype differentiation, and inappropriate expression of \textit{HOXA9} is an early step in EOC that induces aberrant epithelial differentiation. Typically, \textit{HOXA9} is expressed during the development of the reproductive tract and is also overexpressed in breast and ovarian cancer. Additionally, \textit{HOXA9} expression in EOC cells has been shown to induce cancer-related fibroblasts and to provide a microenvironment for tumor growth. Another study showed that \textit{HOXA9} expression promotes tumor growth and stimulated tumor cells to be attached to peritoneal surfaces via inducing expression of the mesenchymal marker P-cadherin. However, the potential roles of \textit{HOXA9} in HGSOC chemotherapy resistance remain incompletely understood.

This study investigated the role of \textit{HOXA9} expression in chemotherapy resistance and the clinical prognosis of HGSOC patients. This is the first study, to our knowledge, to explore the role of \textit{HOXA9} expression in platinum resistance and the clinical prognosis of HGSOC patients.

Materials and methods

Tissue Samples

HGSOC specimens were collected from the Fourth Hospital of Hebei Medical University between July 2010 and June 2017. The application of these specimens for research purposes was approved by the Medical Ethics Committee of the Fourth Hospital of Hebei Medical University (No. 2017ME96), and written informed consent was provided by each study participant. The inclusion criterion were as follows: (1) histologically confirmed primary HGSOC patient tissues of any age; (2) FIGO stage III–IV; (3) patients who received six cycles of platinum-based chemotherapy; and (4) follow-up data of over 5 years was available. The exclusion criteria were: (1) other types of EOC; (2) malignant tumors in other organs; and (3) history of chemotherapy prior to surgery. HGSOC patients were assigned into a chemotherapy-sensitive group (n = 124)
and a chemotherapy-resistant group (n = 80). Platinum-resistance was defined as recurrence less than 6 months from the completion of treatment with platinum-based chemotherapy. Platinum-sensitivity was defined as recurrence in primary HGSOC patients after more than 6 months from the completion of platinum-based treatment. Progression-free survival (PFS) was defined as the interval between the initial surgery to the first disease recurrence or the last follow-up. Overall survival (OS) was defined as the interval between initial surgery and patient death or the last follow-up.

**RNA extraction and quantitative real-time PCR (qPCR)**

Total RNA was extracted from HGSOC tissues using TRIzol reagent (Generay Biotech Co., Ltd., Shanghai, China) in accordance with the manufacturer’s protocols. RNA was used for reverse transcription with the Revert Aid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Target cDNAs were amplified with the SYBR-Green II Premix Kit (Takara, Shiga, Japan) using a two-step amplification procedure on an ABI 7500 detection system (Applied Biosystems, Waltham, MA, USA). The primers used for detecting HOXA9 were as follows: forward 5’-CCCTGACTGACTATGCTTGTGGTTC-3’ and reverse 5’-CTTGTCTCCGCGCTCTTATCATTC-3’. GAPDH was used as an internal control. The primers for GAPDH were forward 5’-ACCACAGTCCATGCCCATAC-3’ and reverse 5’-TCCACCACCCTGTGTCTGTA-3’. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The reaction conditions were as follows: 95°C for 30 s, and then 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 5 s. The relative levels of HOXA9 mRNA expression was calculated according to the cycle threshold (Ct), and the $2^{-\Delta \Delta Ct}$ method was used to measure relative HOXA9 expression.

**HOXA9 immunohistochemistry (IHC)**

HOXA9 IHC was performed on paraffin-embedded HGSOC tissue samples, which were provided by the Fourth Hospital of Hebei Medical University. A primary antibody against HOXA9 (1:200 dilution) was purchased from Proteintech (Wuhan, China).

Briefly, 4-μm-thick HGSOC tissue sections were dewaxed in xylene and dehydrated in a graded ethanol series. Next, 3% H₂O₂ was used to block endogenous peroxidase activity. After eliminating nonspecific binding, the sections were incubated with primary antibody overnight at 4°C, followed by incubation with the corresponding second antibody. Subsequently, tissue sections were stained and re-stained with DAB and hematoxylin, respectively. As a negative control, tissue sections were incubated with PBS rather than primary antibody. HOXA9 staining was positive in tumor cells, showing nuclear expression without cytoplasmic staining. Then, staining was visualized with the CaseViewer scanning system. HOXA9 expression levels were scored according to the staining intensity and proportion of staining. For binary analysis, scores less than 4 were identified as low expression, whereas scores greater than 4 were identified as high expression.

**Kaplan–Meier plotter database**

The Kaplan–Meier plotter tool (http://www.kmplot.com) is an online database that includes survival data for 1104 serous ovarian cancer patients (SOC) and was used to analyze the prognostic value of HOXA9 mRNA expression in SOC patients. To evaluate the prognostic value of HOXA9, SOC samples were stratified into high and low HOXA9 expression groups using the
automatically selected best cutoff. In this study, we analyzed the PFS and OS of SOC patients for over 5 years, including log-rank P values and hazard ratios (HRs) with 95% confidence intervals (CIs). The desired Affy ID (209905_at) was valid in SOC for the Kaplan–Meier plotter database.

**Statistical analysis**

Statistical analyses were performed using SPSS v21.0 (IBM Corp., Armonk, NY, USA). Comparisons of HOXA9 mRNA expression in the two groups were performed using the Wilcoxon rank sum test. Comparisons of HOXA9 protein expression in the two groups were performed using the $\chi^2$ test. Relationships between HOXA9 mRNA expression and the clinical prognosis of HGSOC patients were performed using the Kaplan–Meier plotter and log-rank test. Differences were considered statistically significant at $P < 0.05$ (two-sided).

**Results**

HOXA9 expression was significantly elevated in the chemotherapy-resistant group

To explore the involvement of HOXA9 in platinum-resistance of HGSOC patients, HOXA9 mRNA levels were analyzed by qPCR in 124 chemotherapy-sensitive HGSOC tissues and 80 chemotherapy-resistant HGSOC tissues. The findings revealed 2.30-fold higher HOXA9 mRNA levels in chemotherapy-resistant HGSOC tissues compared with chemotherapy-sensitive HGSOC tissues. These findings indicated significantly higher HOXA9 mRNA expression in chemotherapy-resistant compared with in chemotherapy-sensitive HGSOC patients ($P = 0.002$, Figure 1).

To assess HOXA9 protein expression, we analyzed 80 chemotherapy-resistant HGSOC samples and 124 chemotherapy-sensitive HGSOC samples by IHC. IHC staining revealed that HOXA9 protein was primarily localized to the nuclei of HGSOC tissues (Figure 2a). The frequency of positive HOXA9 expression was significantly higher in the chemotherapy-resistant group than in the chemotherapy-sensitive group ($P = 0.001$, Figure 2b).

**Relationship between HOXA9 expression and the clinicopathological characteristics of HGSOC patients**

To study the relationship between HOXA9 and chemotherapy-resistance in HGSOC patients, we investigated associations of HOXA9 expression with the clinicopathological characteristics of HGSOC patients.

![Figure 1.](image-url) Higher HOXA9 mRNA expression in chemotherapy-resistant HGSOC patients ($P < 0.05$).
No remarkable associations were found between \textit{HOXA9} expression and the FIGO stage or age of HGSOC patients (Table 1). However, high \textit{HOXA9} expression was significantly associated with ascites and residual disease ($P < 0.05$, Table 1).

To further verify the relationship between \textit{HOXA9} mRNA expression and the clinicopathological characteristics of EOC patients, we also observed these relationships using data from The Cancer Genome Atlas (TCGA). No remarkable associations were found between \textit{HOXA9} expression and FIGO stage, age, lymphatic invasion, or histological grade in EOC patients using TCGA data (Table 2).

\textbf{High \textit{HOXA9} expression correlated with poor clinical prognosis in HGSOC patients}

To investigate the impact of \textit{HOXA9} expression on survival in HGSOC patients,
we used the Kaplan–Meier plotter to analyze correlations between HOXA9 mRNA expression and the prognosis of HGSOC patients. Using the median HOXA9 mRNA expression level as the cut-off, the 204 HGSOC patients were divided into low and high HOXA9 mRNA expression groups. The high HOXA9 mRNA expression group was found to be associated with significantly lower OS and PFS than the low HOXA9 expression group ($P = 0.023$, Figure 3a; $P = 0.015$, Figure 3b). To pinpoint the impact of HOXA9 expression on the survival of HGSOC patients, we adjusted other clinical parameters. Univariate and multivariate analyses demonstrated that large residual tumor ($> 1$ cm), ascites ($\geq 1000$ mL), and high HOXA9 expression were independent factors that could predict poor clinical outcomes in HGSOC patients ($P < 0.05$, Table 3).

To further verify the association between HOXA9 mRNA expression and clinical prognosis in HGSOC patients, we also observed their relationship using the Kaplan–Meier plotter database. The median PFS values were 18.23 and 14.67 months for SOC patients with low and high HOXA9 expression, respectively. HGSOC patients with high HOXA9 mRNA expression had shorter PFS (HR = 1.25, 95%CI = 1.06–1.48, $P = 0.007$, Figure 3c) and OS (HR = 1.50, 95%CI = 1.26–1.79, $P < 0.001$, Figure 3d) compared with those with low HOXA9 mRNA expression.

### Discussion

EOC is a fatal cancer in women due to its insidious onset, early metastasis, chemotherapy resistance, and high recurrence rate. HGSOC is the major type of EOC and has a poor prognosis. Although platinum-based chemotherapy regimens can initially be extremely effective in HGSOC patients, the development of chemotherapy resistance has been predominantly responsible for the poor prognosis of this patient population. Thus, understanding the molecular mechanism underlying chemotherapy resistance may provide opportunities for developing novel therapeutic strategies that will improve the prognosis of HGSOC.

In this study, we found significantly higher mRNA and protein expression of HOXA9 in chemotherapy-resistant compared with in chemotherapy-sensitive HGSOC tissues. Additionally, higher HOXA9 expression was associated with

Table 2. Clinical parameters of The Cancer Genome Atlas (TCGA) ovarian cancer (OV) cohort.

| Parameter       | Variable | High (n = 65) | Low (n = 313) | P value |
|-----------------|----------|--------------|---------------|---------|
| Age (years)     | <50      | 13           | 67            | 0.801   |
|                 | $\geq$50 | 52           | 246           |         |
| FIGO stage      | I–II     | 3            | 20            | 0.984   |
|                 | III–IV   | 62           | 293           |         |
| Lymphatic invasion | Yes    | 16           | 84            | 0.917   |
|                 | No       | 49           | 229           |         |
| Histological grade | G1–G2 | 5            | 38            | 0.745   |
|                 | G3–G4    | 60           | 275           |         |
poor clinical outcomes in HGSOC patients, indicating the potential role of HOXA9 in HGSOC patients as an independent prognostic factor.

HOXA9 is dysregulated in most solid tumors, including ovarian, breast, and cervical cancers, and acts as a transcriptional activators to promote tumorigenesis.\textsuperscript{11,14,15}

**Figure 3.** High HOXA9 expression is associated with poor prognosis in HGSOC. (a, b) Kaplan–Meier curves of progression-free survival (PFS) and overall survival (OS) in HGSOC patients categorized by HOXA9 expression and (c, d) the Kaplan–Meier plotter database showed that high HOXA9 expression was significantly associated with reduced PFS and OS in HGSOC patients (P < 0.05).

**Table 3.** Cox analysis of overall survival in HGSOC patients.

| Characteristic        | Univariate analysis | Multivariate analysis |
|-----------------------|---------------------|-----------------------|
|                       | HR                  | 95% CI                | P value | HR    | 95% CI    | P value |
| Age                   | 1.487               | 0.712–2.948           | 0.236   | 1.325 | 0.610–2.657 | 0.476   |
| FIGO stage            | 3.685               | 1.034–5.481           | 0.129   | 3.149 | 1.425–4.879 | 0.289   |
| Residual disease      | 1.656               | 1.281–2.387           | 0.001   | 1.824 | 1.168–2.239 | 0.001   |
| Ascites               | 2.302               | 1.386–4.373           | 0.012   | 1.689 | 1.035–2.778 | 0.014   |
| Serum CA125           | 2.005               | 1.317–5.068           | 0.203   | 1.908 | 1.189–5.985 | 0.072   |
| HOXA9 expression      | 1.084               | 0.982–2.217           | 0.004   | 1.181 | 0.896–2.284 | 0.003   |
HOXA9 is also overexpressed in acute leukemia.\textsuperscript{10} Silencing of HOXA9 is associated with reduced cell proliferation, invasion, and migration, as well as chemoresistance and apoptosis.\textsuperscript{16} Numerous previous studies have reported that HOXA9 is upregulated in malignancies and that high HOXA9 expression is associated with chemotherapy resistance in several malignancies\textsuperscript{17–19} as well as with anti-apoptotic, pro-proliferative, and pro-invasive features.\textsuperscript{20,21} In this study, we confirmed that HOXA9 was overexpressed in HGSOC patients and that HOXA9 expression was increased in chemotherapy-resistant tissues compared with in chemotherapy-sensitive tissues. Thus, these findings provide robust evidence for the pivotal role of high HOXA9 expression in chemotherapy resistance of HGSOC patients. Additionally, our work demonstrated for the first time that high HOXA9 mRNA expression was associated with a low survival rate in HGSOC patients. These findings are in accordance with previous studies in glioblastoma,\textsuperscript{22} head and neck squamous cell carcinoma,\textsuperscript{16} and acute myeloid leukemia.\textsuperscript{23} Furthermore, Céline et al. concluded that altering HOXA9 DNA methylation patterns could be used as a potential prognostic approach for HGSOC.\textsuperscript{24} However, previous studies have not found correlations between high HOXA9 expression and the probability of disease progression or the survival of EOC patients.\textsuperscript{25} Finally, our Kaplan–Meier analyses also suggested an association between high HOXA9 expression and reduced survival in HGSOC patients.

In conclusion, this study clarified that high HOXA9 expression may be of predictive value for platinum resistance and is also an independent marker of poor prognosis in HGSOC patients. Nevertheless, our findings need to be confirmed in a larger patient cohort, which may facilitate new chemotherapy options for HGSOC patients.

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**Declaration of conflicting interests**

The authors have no conflicts of interest to declare.

**Ethical Statement**

The authors are accountable for all aspects of the work and 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).

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