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ORIGINAL ARTICLE

# High expression of herpesvirus entry mediator (HVEM) in ovarian serous adenocarcinoma tissue

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## Summary

***Purpose:*** *To explore the expression of herpesvirus entry mediator (HVEM) in ovarian serous adenocarcinoma tissues and its relationship with clinicopathological features.*

***Methods:*** *Paraffin-embedded specimens from 40 patients with ovarian serous adenocarcinoma who were subjected to surgical treatment were used for the determination of HVEM expression by immunohistochemistry (IHC). Then the relationship between the expression of HVEM and the patient clinicopathological features was analyzed.*

***Results:*** *There were 29 cases (72.5%) of HVEM/tumor necrosis factor receptor (TNFR)SF14-positive and 11 cases (27.5%) of HVEM/TNFRSF14-negative. The positive rate of HVEM was significantly correlated with TNM staging, lymph node metastasis and recurrence ($p<0.05$), but not with age, grade of differentiation and distant metastasis ($p>0.05$).*

***Conclusion:*** *HVEM is highly expressed in ovarian serous adenocarcinoma tissues and correlated with the patient clinicopathological features, such as TNM staging, lymph node metastasis and recurrence. HVEM can provide a basis in the search for a new targeting treatment for ovarian serous adenocarcinoma.*

***Key words:*** *HVEM, immunohistochemistry, ovarian serous adenocarcinoma*

## Introduction

Ovarian serous adenocarcinoma is an invasive epithelial ovarian tumor, a common pathological type with high mortality and low overall 5-year survival, seriously threatening the women's life [1,2]. Although traditional surgery, chemotherapy and radiotherapy can improve patients' condition, the high recurrence rate and drug resistance against chemotherapeutic agents still seriously affect the patient quality of life [3]. Immunotherapy, one of treatment methods for tumors, aims at improving the tumor immunogenicity and the body's immune response to tumor [4]. In recent years, with the development of tumor immunology, it has been found that the treatment method depending just on the tumor antigens and related targets in signal transduction pathways has a number of limitations [5,6], because tumor cells also use a variety of costimulating molecules to achieve immunosuppression in their microenvironment. Herpesvirus entry mediator (HVEM), one member of TNFR/tumor necrosis factor (TNF) family, is also a costimulating molecule belonging to TNF receptor superfamily. Recently, studies at home and abroad have found that the activation of HVEM is involved in the development of many tumors, and closely related to the growth, metastasis and tumors' prognosis [7,8], but HVEM expression in ovarian serous adenocarcinoma tissues and its relationship with the clinicopathological parameters is still unclear. This study was

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conducted to detect the expression of HVEM in 40 cases of ovarian serous adenocarcinoma using IHC and then explored its relationship with the clinicopathological features of ovarian serous adenocarcinoma.

## Methods

*General information*

Paraffin-embedded, formalin-fixed tissue blocks of resected ovarian serous adenocarcinoma from 40 patients (mean age 52.6 years; range 29-76) were retrieved from the histopathological archives of the Department of Pathology in our hospital, from December 2012 to December 2014. This study was conducted in accordance with the declaration of Helsinki and after approval from the Ethics Committee of Tongji University. Written informed consent was obtained from all participants. Tumors were graded into well (N=25), moderately well (N=9), or poorly differentiated (N=6) and typed according to the World Health Organization classification system. The International Union Against Cancer (UICC) staging TNM classification system was used, including 8 cases of stage I, 11 cases of stage II, 15 cases of stage III and 6 cases of stage IV disease. All patients were subjected to transabdominal hysterectomy, bilateral adnexectomy, pelvic adhesions decomposition, greater omentum resection and appendectomy, pelvic lymphadenectomy and abdominal aortic lymphadenectomy. All 40 cases also were administered postoperative chemotherapy with paclitaxel combined with cisplatin or paclitaxel combined with carboplatin with doses according to body surface area and creatinine clearance. Cycles' repetition was every 3 weeks and at least 6 cycles were administered.

*Immunohistochemical method (IHC)*

Paraffin-embedded, 5μm-thick sections were conventionally dewaxed in xylene, rehydrated through graded alcohol, and placed in 3% hydrogen peroxide in methanol for 10 min at room temperature. Then the slides were retrieved by high pressure for 2 min in 0.01M citrate buffer, pH 6.0. After cooling, the slides were incubated with the primary antibody to HVEM/TNFRSF14 (diluted in 1:100, R&D Systems, USA) at 4°C overnight in a humidified chamber, followed by rinse with PBS buffer containing 0.1% Tween-20 for three times. Subsequently, the slides were incubated with polymeric intensifier for 20 min at room temperature, followed by exposure to peroxidase-labeled goat anti-mouse/rabbit antibody (EliVision™ plus System; Fuzhou Maxin Biological Technology Co., LTD., China) for 30 min at room temperature. After that, the immunoreactivity of cells for HVEM/TNFRSF14 was developed by a DAB kit (Fuzhou Maxin Biological Technology Co., LTD., China) and the slides were then counterstained with hematoxylin. A tissue microarray containing ovarian cancer tissues, adjacent tissues and normal ovarian tissues was used as the control for ovarian serous adenocarcinoma. External positive control tissues included ovarian cancer tissues with known positivity for HVEM/TNFRSF14. For negative controls, normal mouse IgG was substituted for primary antibody. The immunohistochemical staining was evaluated by means of light microscopic examination and interpreted by 2 independent pathologists who were blinded to clinical information. The final consensus was discussed and determined in a common session.

*Evaluation of immunostaining*

HVEM/TNFRSF14 immunoreactivity was scored both in the nucleus and cytoplasm. Staining intensity included 4 scales: no staining (scale level 0), weakly positive (dilute brown, scale 1), moderately positive (brown, scale 2) and strongly positive (dark brown, scale 3). According to the positive rate, staining also included score 0 for the area <1%, score 1 for 1-24%, score 2 for 25-49%, and score 3 for 50-100%. At least 5-10 high power fields (HPF) were randomly observed. Finally the immunoreactivity of each sample for HVEM/TNFRSF14 was determined by the average of the comprehensive evaluation of intensity and positive rate.

*Statistics*

SPSS17.0 software was used to process data in this study. The positive rates of HVEM between groups were compared using chi-square test. A p value <0.05 was considered as statistically significant.

## Results

*Expression of HVEM/TNFRSF14 in ovarian cancer tissues, adjacent tissues and normal tissues*

No stain was found in the negative control, but obvious dark brown was found in the positive control, which was specific and clear (Figure 1). The ovarian serous adenocarcinoma tissues in TNM stage I showed oval nuclei, even chromatin distribution, little heteromorphism and HVEM-positive in nuclei (+) (Figure 1-A1). Figure 1-A2 represents the negative control for Figure 1-A1. The ovarian serous adenocarcinoma tissues in TNM stage III showed HVEM-positive in nuclei and the cytoplasm (+++) and high heteromorphism, inhomogenous nuclei, thick chromatin, a lot of karyokinesis, and some giant cells (Figure 1-B1). Figure 1-B2 represents the negative control for Figure 1-B1. Figure 1-C1 showed HVEM-positive in the nuclei and cytoplasm (++) of ovarian serous adenocarcinoma in TNM stage II. Figure 1-C2 represents the negative control for Figure 1-C1. TNM stage II ovarian serous adenocarcino-

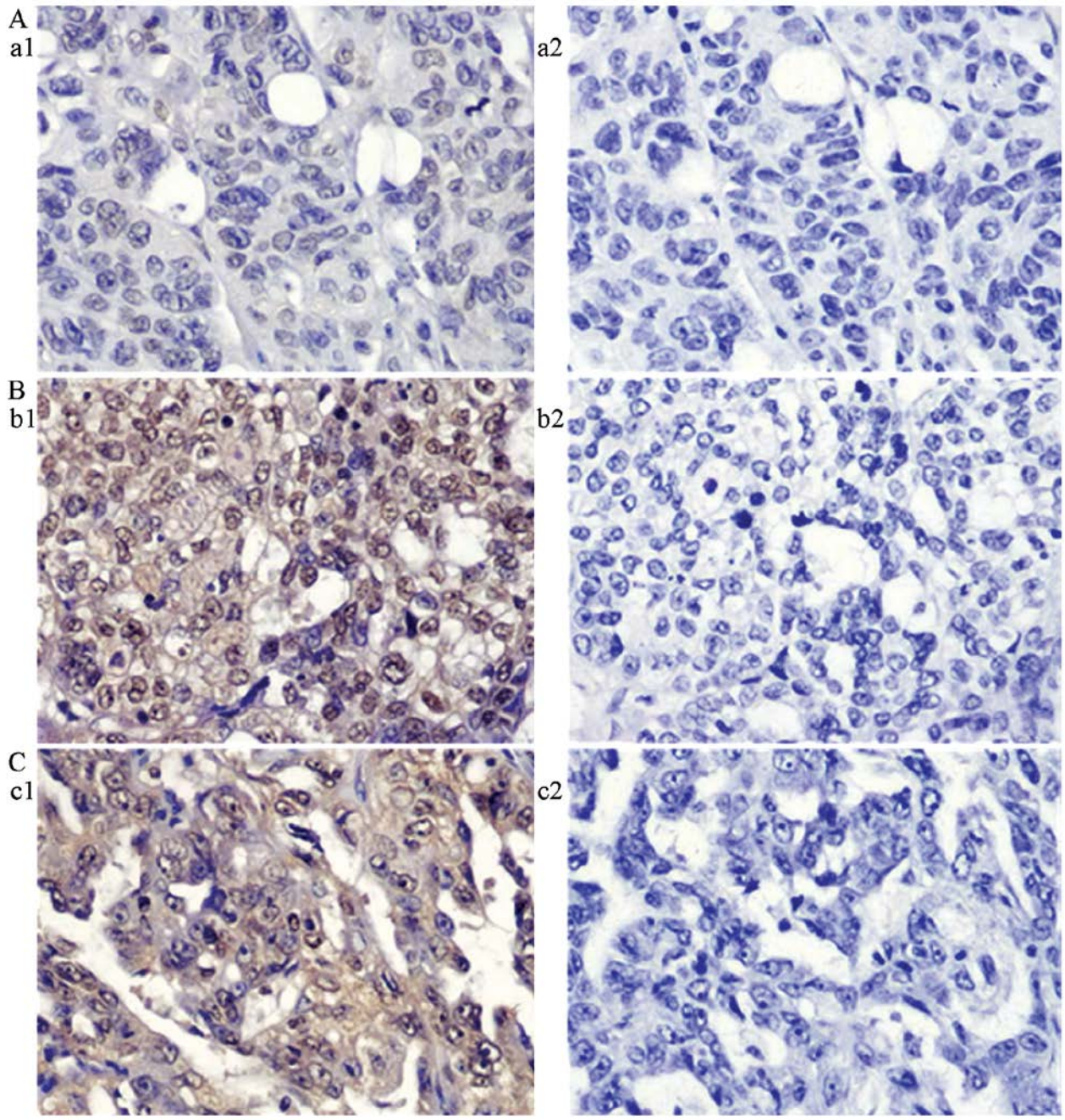


**Figure 1.** Expression of HVEM/TNFRSF14 in ovarian serous adenocarcinoma and their negative controls (x200). Ovarian serous adenocarcinoma tissues in TNM stage I show oval nucleus, even chromatin, little heteromorphism and HVEM-positive in nuclei (+) (Figure 1-A1). Figure 1-A2 is negative control for Figure 1-A1. Ovarian serous adenocarcinoma tissues in TNM stage III show HVEM-positive in nuclei and cytoplasm (+++) and high heteromorphism, inhomogeneous nuclei, thick chromatin, high karyokinesis, and some giant cells (Figure 1 - B1). Figure 1-B2 is negative control for Figure 1-B1.
Figure 1-C1 shows HVEM-positive in the nuclei and cytoplasm (++) of ovarian serous adenocarcinoma in TNM stage II. Figure 1–C2 is negative control for Figure 1-C1.
Immunoreactivity detection of cells for HVEM/TNFRSF14 was done by DAB kit.

ma also revealed obvious heteromorphism with occasional karyokinesis.

*Expression of HVEM/TNFRSF14 in 40 cases of ovarian serous adenocarcinoma*

Among the 40 cases of ovarian serous adenocarcinoma tissues, there were 29 (72.5%) cases of HVEM/TNFRSF14-positive, and 11 (25.5%) cases of HVEM/TNFRSF14-negative. Histopathological examination revealed less heteromorphism, oval nuclei and even chromatin distribution in stage I ovarian serous adenocarcinoma (Figure 2-A). More heteromorphism, inhomogeneous nuclei and some karyokinesis were seen in stage II ovar-

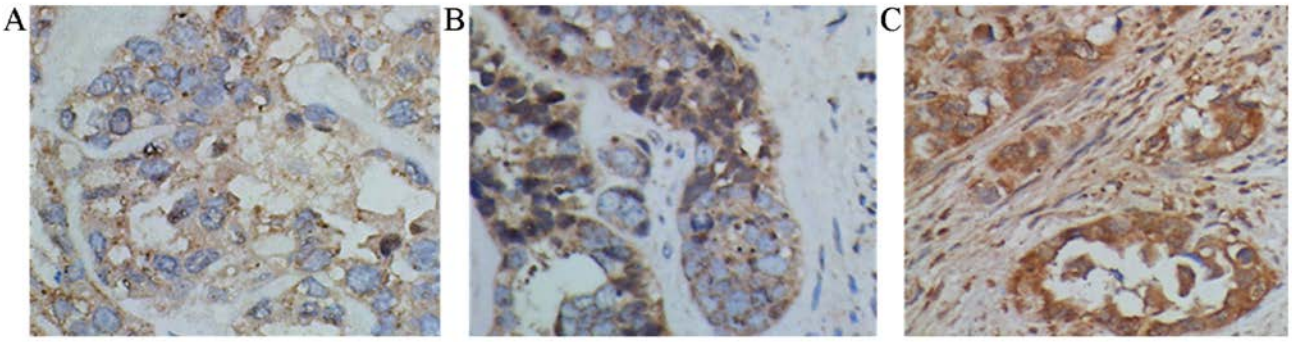


**Figure 2.** Expression of HVEM/TNFRSF14 in ovarian serous adenocarcinoma tissues (×200). Histopathological examination reveals less heteromorphism, oval nuclei and even chromatin in stage I ovarian serous adenocarcinoma (Figure 2-A), more heteromorphism, inhomogeneous nuclei and some karyokinesis in stage II ovarian serous adenocarcinoma (Figure 2-B) and obvious heteromorphism and more karyokinesis in ovarian serous adenocarcinoma stage III (Figure 2-C). Immunoreactivity detection of cells for HVEM/TNFRSF14 was done by DAB kit.

**Table 1.** Relationship between HVEM/TNFRSF14 expression in ovarian serous adenocarcinoma tissues and patient clinicopathological characteristics

| *Clinicopathological characteristics* | *Number of cases* | *HVEM/TNFRSF14* | | $x^2$ | *p value* |
|---|---|---|---|---|---|
| | | Positive | Negative | | |
| Age (years) | | | | | |
| >50 | 26 | 19 | 7 | 1.36 | 0.369 |
| <50 | 14 | 10 | 4 | | |
| Differentiation | | | | | |
| Well | 25 | 21 | 4 | 1.57 | 0.228 |
| Moderate, Poor | 15 | 8 | 7 | | |
| TNM stage | | | | | |
| I+II | 19 | 11 | 8 | 5.69 | 0.003 |
| III+IV | 21 | 18 | 3 | | |
| Lymph node metastasis | | | | | |
| No | 17 | 11 | 6 | 7.34 | 0.016 |
| Yes | 23 | 18 | 5 | | |
| Distant metastasis | | | | | |
| No | 21 | 16 | 5 | 2.33 | 0.376 |
| Yes | 19 | 13 | 6 | | |
| Recurrence | | | | | |
| No | 24 | 16 | 8 | 8.26 | 0.008 |
| Yes | - | - | - | - | - |

ian serous adenocarcinoma (Figure 2-B) and obvious heteromorphism and more karyokinesis in stage III ovarian serous adenocarcinoma (Figure 2-C).

*Relationship between expression of HVEM/TNFRSF14 and clinicopathological features of ovarian serous adenocarcinoma*

To confirm whether the expression of HVEM/TNFRSF14 in ovarian serous adenocarcinoma tissues is associated with the clinicopathological features, we analyzed the relationship between the expression of HVEM/TNFRSF14 and the patient clinicopathological features. The results showed that the positive rate of HVEM/TNFRSF14 in patients with TNM stage III and IV was significantly higher than that in patients with TNM stage I and II ($p<0.05$, Table 1). It was also remarkably higher in patients with lymph node metastasis than in patients without lymph node metastasis ($p<0.05$). Furthermore, the positive rate in patients with recurrence after surgery was significantly higher compared with patients without recurrence ($p<0.05$). All the above results suggested that HVEM/TNFRSF14 expression was associated

with TNM staging, lymph node metastasis and recurrence. However, the expression of HVEM/TNFRSF14 didn't show any relationship with age, grade of differentiation and distant metastasis.

## Discussion

In the last 40 years, the incidence of ovarian cancer increased 2 to 3 times and has a gradually rising trend [9]. According to a recent report in 2007 from the American Cancer Society, about 15280 patients with newly diagnosed ovarian cancer died of ovarian cancer [1], showing its high mortality rate which is on top of gynecological malignancies and seriously threatening women's health and life [10]. Traditional therapeutic methods (surgery, chemotherapy and radiotherapy) may ameliorate the disease's condition, but also bring a number of problems such as high recurrence rate, resistance against chemotherapeutic agents and lowering the patient quality of life [11].

HVEM, the first member in tumor necrosis factor receptor superfamily that is able to interact with immunoglobulin superfamily member, was firstly found by Montgomery et al. when they screened the genes mediating herpes simplex virus entering Chinese hamster ovary (CHO)-K1 cell [12]. HVEM is highly expressed in tissues and organs rich in lymphocytes, such as spleen and the lymph nodes. It is also constitutively expressed on peripheral T, B cells, monocytes and immature DC cells [13,14]. $CD4^+$ and $CD25^+$ regulatory T cells have four extracellular cysteine-rich domains (CRD). When it is triggered by antigen, the increased HVEM combines with the negative costimulating molecule BTLA via CRD1 and inhibits lymphocyte activation signals [15,16], inhibiting thus the function of effector T cells [17]. As a ligand, HVEM unidirectionally transmits inhibitory signals to BTLA to inhibit T cell activation and proliferation, resulting in disease deterioration and suggesting that it is associated with the expression of HVEM [18].

This study was conducted to explore if the incidence and process of ovarian serous adenocarcinoma is associated with the expression of HVEM in cancer tissues, so as to identify the impact of the changes of HVEM expression on these factors. Our results showed that there were 18 cases of HVEM/TNFRSF14-positive in TNM stage III and IV and 11 cases in stage I and II. The expression of HVEM/TNFRSF14 in TNM stage III and IV was significantly higher than that in TNM stage I and II ($p<0.05$), suggesting HVEM/TNFRSF14 is associated with TNM staging. We also detected HVEM/TNFRSF14 expression in ovarian cancer tissues by IHC and found that the expression of HVEM/TNFRSF14 in ovarian serous adenocarcinoma was similar to that in most of ovarian cancers. The expression of HVEM/TNFRSF14 in stage III ovarian serous adenocarcinoma was significantly higher than that in stage II and I. In addition, the results showed that the expression of HVEM/TNFRSF14 was significantly higher in patients with lymph node metastasis compared with those without lymph node metastasis ($p<0.05$), and the positive rate of HVEM/TNFRSF14 in patients with recurrence after surgery was significantly higher than that in patients without recurrence ($p<0.05$).

It is speculated that the mechanism for the correlation of HVEM with tumor invasion and metastasis always lies in the immune tolerance state in the systemic and local immune system [19,20]. HVEM inhibits the function of effector T cells via binding to the costimulating molecule involved in the regulation of lymphocytes' activation, and thus causing a physiological state of immune tolerance [21,22]. Our previous study had established an animal tumor model by using SCID mice and found that the expressions of BTLA and HVEM were both increased in the tumor microenvironment and the secretion of interleukin (IL)-2 and interferon-γ (IFN-γ) were reduced, inhibiting thus the upregulation of transforming growth factor (TGF)-β and IL-10 and maintaining the tumor in a state of local immunosuppression [23]. Our previous work also found that anthropogenic skov3, C13K, coc1 and other ovarian cancer cell lines and ovarian epithelial carcinoma tissues had high expression of HVEM. It has been shown that tumor cells can express some ligands for common costimulating molecules to induce abrogation of effector T cells in the tumor microenvironment, so as to escape from immune surveillance and accelerate the deterioration of disease [24-26].

From the expression of HVEM/TNFRSF14 in a micro tissue array of ovarian cancer we revealed that HVEM/TNFRSF14 was highly expressed in ovarian serous adenocarcinoma tissues, both in nucleus and the cytoplasm (Figure A1, B1, C1). According to the pathology results, tissues with weakly positive HVEM/TNFRSF14 expression (+) belonged to TNM stage I, showing oval nuclei, even chromatin distribution and less heteromorphism. Tissues with strongly positive HVEM/TNFRSF14 (+++) belonged to TNM stage III, showing significant cell atypia, inhomogeneous nuclei and more karyokinesis. In this study, IHC technique was applied to analyze the HVEM expression in

40 cases of ovarian serous adenocarcinoma tissues, 29 (72.5%) cases of which were HVEM/TNFRSF14 positive, and 11 (27.5%) cases were negative, further suggesting that the increased HVEM expression was associated with the disease grade and clinical stage.

To sum up, in this study, we observed the HVEM expression in ovarian serous adenocarcinoma tissues and explored its correlation with clinicopathological characteristics to investigate the pathogenesis of this disease to possibly reveal a new molecular mechanism and provide a theoretical basis for the development of new targeted therapeutic approaches.

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## Conflict of interests

The authors declare no confict of interests.

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