

Original Article

Loss of expression of EphB1 protein in serous carcinoma of ovary associated with metastasis and poor survival

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Abstract: Aberrant expression of receptors tyrosine kinase of Eph gene in human cancers is extensively documented. We previously found that EphB1 subtype is down-regulated in gastric cancer and colorectal cancer. For the more, decreased expression of EphB1 is related to invasion and metastasis in cancers. There is no published data regarding the role of EphB1 in ovarian cancer, which is the focus of the present study. The expression of EphB1 protein was determined in tissues from 74 patients with serous ovarian carcinoma and 12 normal ovarian epithelial tissues. The expression level of EphB1 protein in serous ovarian carcinoma was analyzed with respect to clinicopathological parameters and survival. EphB1 protein was positively stained in 12 normal ovarian epithelial samples, and negatively stained in 32 out of 74 (43.2%) serous ovarian cancers. Loss of expression of EphB1 protein was associated with higher tumor grade ($P = 0.006$), metastasis ($P = 0.049$) and high proliferative index Ki67 expression ($P = 0.022$), but not with FIGO stage ($P = 0.0937$), age at diagnosis ($P = 0.624$), and diameter of carcinoma ($P = 0.108$). In addition, loss of EphB1 protein in serous ovarian carcinoma was associated with a significantly worse overall survival ($P = 0.015$). Our data indicate that loss of EphB1 protein is associated with metastasis and poorer survival in patients with serous ovarian cancer. EphB1 may be used as a prognostic marker and a therapeutic target in serous ovarian carcinoma.

Keywords: EphB1, serous ovarian carcinoma, metastasis, survival

Introduction

Ovarian carcinoma is the leading cause of death from gynecologic cancer and remains the most common cancer diagnosis in females. Ovarian carcinoma includes several histological types and serous ovarian carcinoma is the most common subtype of epithelial ovarian cancer that accounts for approximately 50-60% of all ovarian cancers [1-4]. Because of a lack of early warning signs and vague symptomatology, most ovarian cancer patients present with widespread metastatic disease. Although improvements in surgical management and advances in cytotoxic therapy have been accomplished in the past decades, the overall 5-year survival rate for women with advanced disease can be as low as 13%. Though a number of tumor markers have been evaluated for monitoring response to treatment and progno-

sis of ovarian cancer, these are still insufficient. Thus, there is an important need for additional diagnostic and prognostic markers for this disease [5-11].

The erythropoietin producing hepatocellular carcinoma (Eph) subfamily of genes encoding receptor tyrosine kinases are structurally characterized by the juxtaposition of a vestigial immunoglobulin-like domain, a single cysteine-rich region, and two FN III domains in the extracellular region. The Eph receptors comprise eight EphA and six EphB receptors based on the similarity within each group of the extracellular domain sequences and on the affinity for binding ephrin-A and -B ligands. Eph receptors and their ligands of ephrin form a large family of receptor tyrosine kinases that involved in several physiological and pathological processes [12-16]. EphAs are typically bound to ephrinAs

via a glycosylphosphatidylinositol anchor on the cell membrane. EphBs are typically bound to ephrinBs via a transmembrane domain. There is a great diversity of ligands and receptors that have tissue-specific expression patterns and overlapping ligand-receptor specificities. Because both the Eph receptors and ephrins localize to the cell surface, the signaling is restricted to the sites of direct cell-to-cell contact. A key feature of these interactions is that their bi-directional signaling is triggered by ligand-receptor interactions and consequent receptor dimerization and the formation of higher-order clusters of activated receptors. The physiological role of Eph receptors is crucial in embryonic developmental processes, such as cell migration, vascular development, tissue border formation, axonal and synaptic network development.

Apart from development, emerging data also suggest the potential involvement of Eph receptors in tumorigenesis [17-21]. Eph-ephrin signaling has the multi-faceted functions as tumor promoters, tumor suppressors, angiogenic inducers and regulators of stem cell homeostasis. In our previous study on the Eph family, we have found that the reduced expression of EphB1 in colorectal cancer more often occurred in poorly differentiated and mucinous adenocarcinomas and showed more invasive power [22]. Interestingly, we also have found that underexpression of EphB1 protein is significantly associated with invasive, advanced stage and metastasis in gastric cancer [23]. To date, there have been no published reports evaluating the role of EphB1 expression in ovarian carcinomas, particularly with respect to clinical outcome. We examined the expression of EphB1 protein in a series of surgically treated serous carcinomas of ovary. The current study was undertaken with the aim to assess the expression of EphB1 protein and its correlation with clinicopathological variables and survival.

Materials and methods

Patients and clinicopathological variables

Archival paraffin-embedded tissue blocks from 74 patients (range 22-79, mean 52 years) with serous cancer of ovary were obtained from Jinling Hospital, Nanjing, China, between the years 2001 and 2010. All hematoxylin-and

eosin-stained slides were re-reviewed by a gynecological pathologist to verify the diagnosis, histological grade, and stages. Women with other histological subtypes were excluded from present study. Pathological stage and histological subtype were determined for each surgical specimen according to 2002 International Federation of Gynecology and Obstetrics (FIGO) criteria, and Pathology and Genetics Tumors of the Breast and Female Genital Organs (World Health Organization, WHO 2003). Patient data were obtained from hospital tumor registry and chart review. All cases of recurrence had radiographic evidence of disease or biopsy proven progression of disease. Ethical approval was obtained from Jinling Hospital Ethics Board. Follow-up ranged from 3-143 months. The records of patients who were alive at follow-up or who did not die of disease were considered to be censored.

Immunohistochemistry

Sections from surgical specimens had been fixed in 10% formalin and embedded in paraffin and they were used here for immunohistochemical staining according to a standard method. Briefly, each 4- μ m tissue section was deparaffinized and rehydrated. After rehydration through a graded ethanol series, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min for antigen retrieval, then cooled to 30°C and washed with phosphate-buffered saline (PBS, pH 7.3). After endogenous peroxidase had been quenched with aqueous 3% H₂O₂ for 10 minutes and washed with PBS, the sections were incubated at 4°C overnight with an EphB1 polyclonal antibody (Abgent, San Diego, CA, USA) at a 1:100 dilution in antibody diluent solution (Zymed, Invitrogen) and then washed with PBS. Next, the sections were incubated with secondary antibody (Dako REAL EnVision Detection System, Dako, UK) for 30 min at room temperature. Color development was performed with 3, 3'-diaminobenzidine (DAB). Nuclei were lightly counterstained with hematoxylin. Two pathologists independently assessed the immunostained slides. Any difference in immunohistochemical scores was resolved by a consensus. Immunohistochemical staining of cancer cells was semi-quantitatively assessed according to the staining intensity and percentage of positive cells. EphB1 expression was assessed for intensity (0 = no staining, 1 = weak, 2 = moder-

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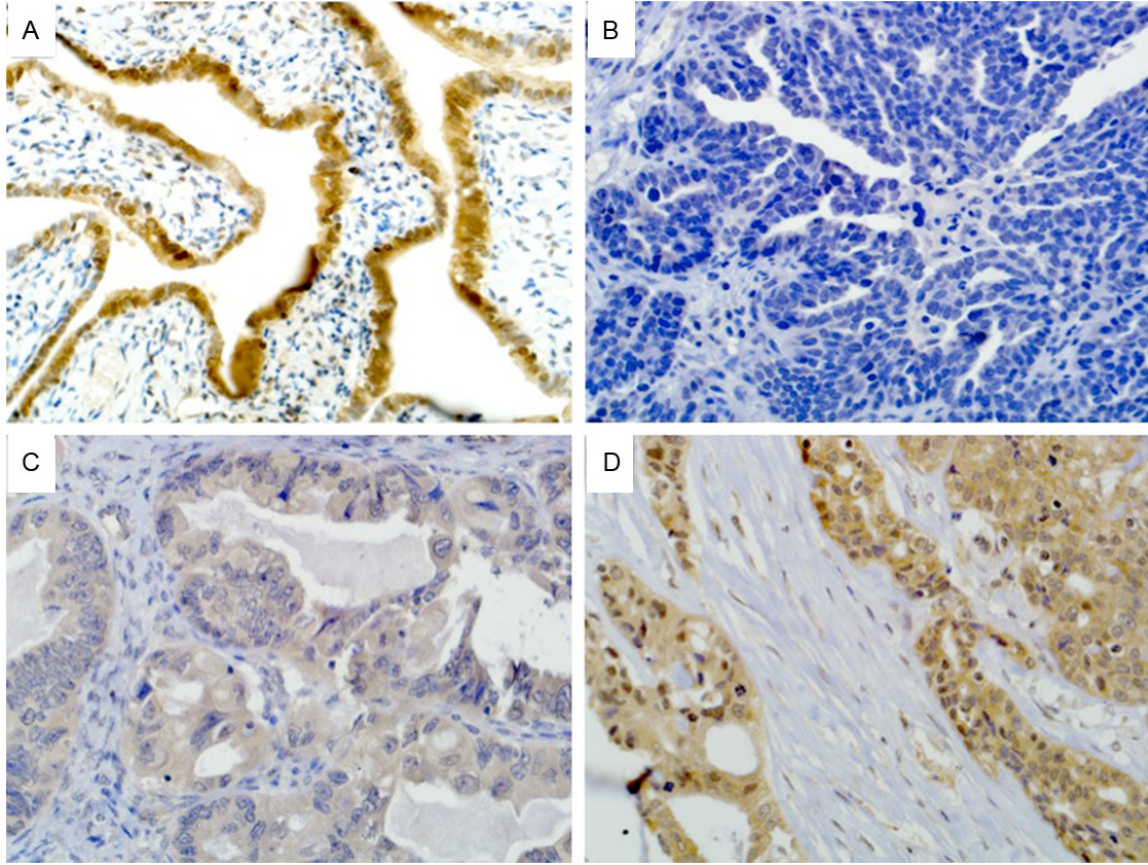


Figure 1. Expression of EphB1 in normal ovarian epithelial and serous ovarian carcinoma. A: Positive staining of EphB1 in normal serous epithelium of fallopian tube cells. B: Negative staining of EphB1 in serous ovarian carcinoma. C: Weak staining of EphB1 in serous ovarian carcinoma. D: Strong staining of EphB1 in serous ovarian carcinoma.

ate, 3 = strong) and the percentage of positive cells (0 = 0%, 1 ≤ 10%, 2 = 10% to 50%, 3 = 51% to 80%, 4 ≥ 80% positive cells) as defined previously. The scores for intensity and percentage were multiplied and a cut-off of 6 was used. Here, we used colorectal cancer and normal mucosa tissues that showed negative and positive expression of EphB1 as controls. The specificity of EphB1 antibody was investigated by using blocking peptide as we previously reported [22]. The percentage of tumor cells positive for Ki-67 was considered low, moderate and high when <10%, 10-60, and >60% of tumor cells, respectively, showed positive staining.

Statistical analysis

The statistical significance of intergroup differences was evaluated by a chi-square test. Kaplan-Meier survival curve was calculated using breast cancer-related death (overall survival) as the endpoints. All statistical analyses were performed using SPSS software (SPSS

16.0, Chicago, IL). A two-sided *P* value of less than 0.05 was considered statistically significant.

Results

Clinicopathological characterization

The mean patients age at the time of diagnosis was 52 years (range 22-79 years). Lymph node sampling or dissection was performed for 46 patients (62%) with 30 patients (65%) demonstrating lymph node metastasis. Eight (11%) and 6 (8%) patients were diagnosed in FIGO stage I and II, respectively, while 49 (66%) patients had FIGO stage III and 11 patients (15%) presented with metastatic disease (FIGO IV). Histological classification was performed according to the World Health Organization system in well-differentiated (G1; *n* = 10), moderately differentiated (G2; *n* = 11), and poorly differentiated (G3; *n* = 53).

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Table 1. Correlation of clinicopathological variables with EphB1 expression in patients with serous ovarian cancer

Variable	EphB1 expression		P Value
	Positive (n = 42)	Negative (n = 32)	
Stage			
I	4	4	0.093
II	4	2	
III	28	21	
IV	6	5	
Grade			
1	8	2	0.006
2	10	1	
3	24	29	
Low	34	31	0.069
High	8	1	
Diemeter (cm)			
≤5	19	11	0.108
5-10	10	15	
≥10	13	6	
Age (years)			
<50	13	12	0.624
≥50	29	20	
Recurrence			
Yes	17	14	0.815
No	25	18	
Metastasis			
Yes	17	13	0.049
No	14	2	
Not available	11	17	
Ki-67			
≤10%	8	6	0.022
10%-60%	20	6	
≥60%	14	20	

EphB1 protein expression in normal ovarian epithelium and in serous ovarian carcinoma

The EphB1 protein was localized in the cytoplasm of the epithelial cells. The cytoplasm of normal ovarian surface epithelium and the normal serous epithelium of fallopian tube cells stained strongly for EphB1 in 12 samples (**Figure 1A**). Expression of EphB1 was immunostained in 74 patients with serious ovarian carcinoma. The staining level of EphB1 protein was varied among serous ovarian carcinoma samples, showing as negative, weak, moderate and strong staining of EphB1 (**Figure 1B-D**). The scores for intensity and percentage of carcinoma cells with positive staining of EphB1 were multiplied and a cut-off of 6 was used. The

EphB1 protein was negatively stained in 32 out of 74 (43%) serous ovarian cancers.

Association between EphB1 expression and clinicopathological parameters

Table 1 shows the association between EphB1 expression and clinicopathologic parameters. Loss of expression of EphB1 is more often detected in serious ovarian carcinomas with high grade ($P = 0.006$) and metastasis ($P = 0.049$). No relationship was found between expression of EphB1 and tumor stage ($P = 0.0937$), diameter ($P = 0.108$), age ($P = 0.624$), and recurrence ($P = 0.815$).

Decreased expression of EphB1 is related high cell proliferation index of Ki-67

Ki-67, a nuclear marker of cell proliferation, is associated with worse outcomes in certain carcinomas. In the present study, we analyzed the relation between expression of EphB1 and Ki-67 protein. Ki-67 immunopositivity was observed in tumor cell nuclei. The median percentage of Ki-67-positive tumor cell nuclei was 45.5% (range, 1% to 90%). High proliferation (>60%) was observed in 33.3% of tumor samples with positive expression of EphB1, while in 62.5% of tumor samples with negative expression of EphB1 (**Table 1**). Loss expression of EphB1 is positively related to high cell proliferation index of Ki-67 ($P = 0.022$).

Loss of expression of EphB1 protein is correlated to a poor overall survival

Seventy-four cases stained for EphB1 had follow-up data for survival and were available for assessment. At the end of the follow-up period, 34 (45.9%) patients were dead of their ovarian carcinoma. Decreased expression of EphB1 was significantly associated with poor overall survival (**Figure 2**, $P = 0.015$).

Discussion

The receptor tyrosine kinases (RTKs) have well-established roles in both normal physiology and oncogenesis. Eph receptors, the largest subfamily of receptor tyrosine kinase, were

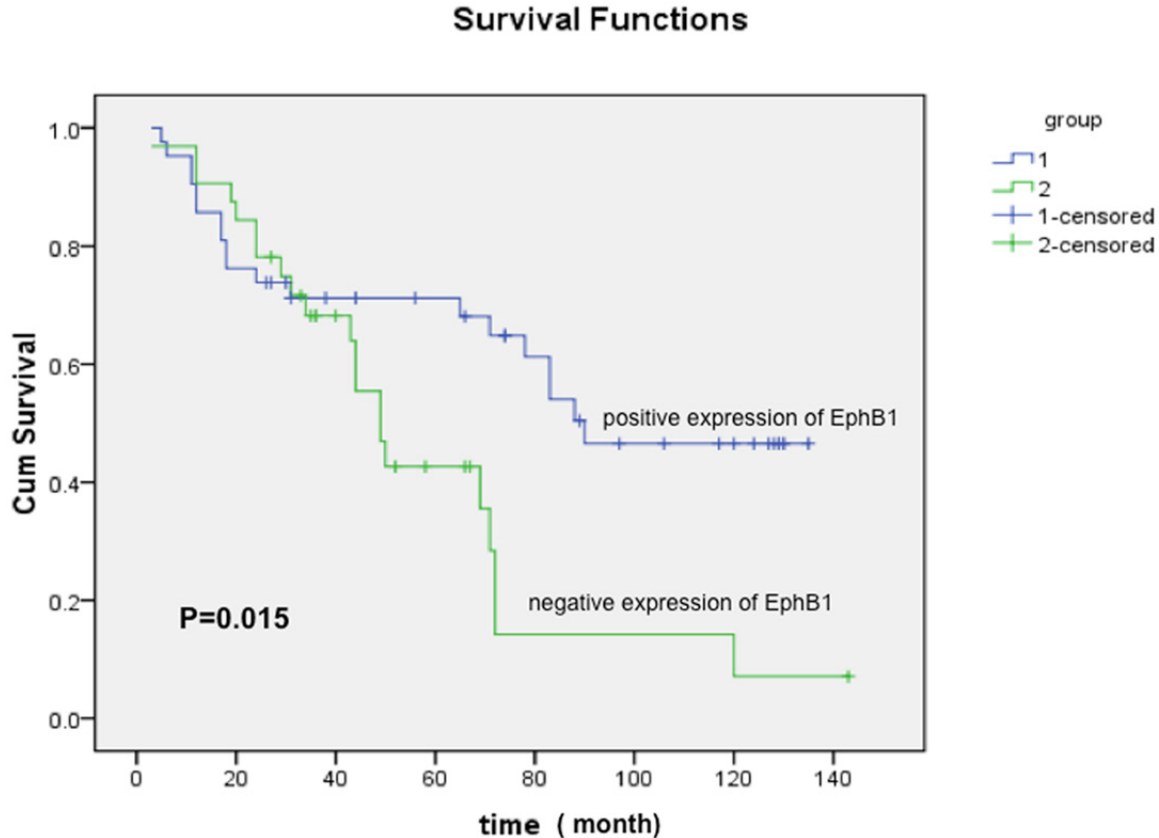


Figure 2. Survival curve of EphB1 immunoreactivity for serous ovarian carcinoma patients. Patients with carcinoma loss of expression of EphB1 showing poor survival, $P = 0.015$.

primarily considered as a classical oncogene. The overexpression of EphA2 receptor is well documented in many human cancers including prostate cancer [24], colorectal cancer [25], gastric cancer [26], and ovarian cancer [27]. Elevated EphA2 expression is correlated with disease stage, increased tumor metastasis, and poor patient survival. EphA2 plays a critical role in cancer progression. EphA2 is frequently overexpressed in non-small cell lung cancer. High level of EphA2 in the primary tumor predicts brain metastasis [28]. Huang et al found that overexpression of EphA2 in gastric cancer cells resulted in the upregulation of the epithelial-mesenchymal transition (EMT) molecular marker N-cadherin and Snail, as well as the Wnt/beta-catenin targets TCF4, CyclinD1, and c-Myc. These demonstrate that EphA2 upregulation is a common event in gastric cancer specimens that is closely correlated with cancer metastasis and that EphA2 promotes EMT of gastric cancer cells through activation of Wnt/beta-catenin signaling [29]. EphB2 is overexpressed in gastric cancer and colorectal can-

cer, and could be used as a prognostic factor and a therapeutic antibody drug target for the cancer treatment [30-32].

However, the role of Eph receptors and Ephrin ligands in oncogenesis is apparently complex and remains ill-defined. There are increasing conflicted data regarding Eph receptors in different cancer types, especially on its putative function as an oncogene or a tumor suppressor gene. Expression of EphA3 lost in human T-cell line HPB-ALL is associated with the hypermethylation of 5' upstream CpG island [33]. Recently, research results from the EphB2 and EphB4 in colorectal cancer have strengthened that Eph receptor can be a tumor suppressor gene [30, 34]. Our group previously reported that down-regulation of the EphA7 receptor in colon cancer cell lines and colorectal cancer samples are secondary to hypermethylation of the 5'CpG island, and down-regulation of the EphA7 is related to differentiation of colorectal cancers [35]. The expression of EphA7 in gastric carcinomas showed that EphA7 was decreased in all

tested gastric cancer cell lines, but exhibited marked variability in gastric carcinoma specimens. Overexpression of EphA7 was more frequently observed in younger patients and in patients with advanced stage, and there is no significant relation between the expression of EphA7 and differentiation in gastric carcinoma [36]. These data indicated that Eph receptors carry unique functions depending on the organ or cell lineage.

EphB1 is a member of the Eph receptor tyrosine kinase family shown to have very important roles in nervous and vascular system development. It is a marker expressed in venous endothelial cells throughout embryonic development to adulthood. The forward signaling by EphB1/EphB2 interacting with ephrin-B ligands at the optic chiasm is required to form the ipsilateral projection [37, 38], and EphB1 forward signaling in the spinal cord plays critical roles in the development of bone cancer pain and morphine tolerance in treating bone cancer pain [39, 40]. In contrast to these more established roles, EphB1's function in cancer is much less clear. Previously, we detected EphB1 transcript and protein in colorectal cancer and gastric cancer [41, 42]. EphB1 transcript and protein were differentially expressed in gastric and colorectal cancer cells among specimens. Reduced expression of EphB1 in colorectal cancers more often occurred in poorly differentiated and mucinous adenocarcinomas than in well- and moderately differentiated adenocarcinomas. Colorectal cancer cells with a low level of EphB1 protein showed more invasive power. In gastric cancer, we found that loss of expression of EphB1 protein is significantly associated with invasion, advanced stage and metastasis. In the present study, we show that EphB1 expression is associated with patient outcome in a series of consecutive cases of serous ovarian cancer specimens ($n = 74$). Loss of EphB1 protein was shown in 43.2% serous ovarian cancers. To our knowledge EphB1 expression has not been previously studied in malignant ovarian tissue. Loss of expression of EphB1 was associated with higher tumor grade ($P = 0.006$) and metastasis ($P = 0.049$), but not with stage ($P = 0.0937$), age ($P = 0.624$), and diameter ($P = 0.108$). In addition, loss expression of EphB1 is positively related to high cell proliferation index of Ki-67 ($P = 0.022$), and is associated with a significantly worse survival ($P = 0.015$).

Our data indicate that loss of EphB1 protein is associated with metastasis and poorer survival in patients with serous ovarian cancer. EphB1 may be used as a prognostic marker and a therapeutic target in serous ovarian carcinoma.

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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