

## RESEARCH ARTICLE

**EphB1 and Ephrin-B, New Potential Biomarkers for Squamous Cell/adenosquamous Carcinomas and Adenocarcinomas of the Gallbladder**Yuan Yuan<sup>1,2</sup>, Zhu-Lin Yang<sup>2\*</sup>, Xiong-Ying Miao<sup>2</sup>, Zi-Ru Liu<sup>2</sup>, Dai-Qiang Li<sup>3</sup>, Qiong Zou<sup>1,2</sup>, Jing-He Li<sup>4</sup>, Lu-Feng Liang<sup>5</sup>, Gui-Xiang Zeng<sup>6</sup>, Sen-Lin Chen<sup>7</sup>**Abstract**

Squamous cell/adenosquamous carcinoma (SC/ASC) of the gallbladder are rare tumors and there are few clinical reports in the literature. Herein we report our clinical experience with 46 patients with SC/ASC and 80 with adenocarcinoma (AC). Expression of EphB1 and Ephrin-B in each tumor was determined using immunohistochemical methods for determination of correlations with prognosis. There was no difference in EphB1 and Ephrin-B expression between SC/ASC and AC tumors ( $P > 0.05$ ), but greater expression in those less than 3 cm in diameter, stage I or II (TNM stage), with no lymph node metastases, with no local invasion and treated with radical resection was apparent. Expression of EphB1 ( $P < 0.05$ ) and Ephrin-B ( $P < 0.01$ ) was higher in well differentiated than in poorly differentiated AC tumors. Kaplan-Meier survival analysis indicated that degree of differentiation, tumor diameter, lymph node metastases, local invasion, surgical approach and expression rate of EphB1 and Ephrin-B were closely related to the survival of SC/ASC ( $P < 0.05$ ) and AC patients ( $P < 0.01$ ). Patients with tumors that positive expressed EphB1 and Ephrin-B, whether it is SC/ASC ( $P_{SC/ASC} = 0.000$ ) or AC ( $P_{AC} = 0.000$  or  $P_{AC} = 0.002$ ) had longer survival than those negative expression. Cox multivariate analysis indicated a negative correlation between expression of EphB1 or Ephrin-B and overall survival. Hence, EphB1 and Ephrin-B could be regarded as independent good prognostic factors and important biological markers for SC/ASC and AC of gallbladder.

**Keywords:** Gallbladder SC - gallbladder ASC - gallbladder AC - EphB1 - Ephrin-B - immunohistochemistry

*Asian Pac J Cancer Prev*, **15** (3), 1441-1446

**Introduction**

Gallbladder cancer is the most common malignant tumor found within the biliary tract (Kapoor, 2006), and the fifth most common of the digestive tract cancers (Kumar et al., 2006; Kayaharan and Nagakawa, 2007; Kalita et al., 2012). Primary Gallbladder Carcinoma refers to malignant tumors that originate from gallbladder epithelial cells. Adenocarcinoma (AC) accounts for 90% of primary gallbladder cancers. Squamous carcinoma and adenosquamous carcinoma of the gallbladder are rare pathologic subtypes, with an incidence of 1.4%-10.4% (Kim et al., 2011; Hamdani et al., 2012). Chan et al. reported 14 gallbladder SC/ASC cases and believed that the clinical features were similar to that of AC, and patients frequently presented with advanced cancer stage and had a poor prognosis (Chan et al., 2007). The main aim of this paper was to identify molecular markers for

the early diagnosis and staging of AC and SC/ASC.

As the biggest subfamily of receptor tyrosine kinases, Eph is widely expressed in cells and the structure is highly conserved. According to the new nomenclature proposed in 1996 (Eph Nomenclature Committee, 1997), the Eph receptor was categorized into EphA and EphB by differences in homologous sequences and their ligand was also separated into Ephrin-A and Ephrin-B. Since Ephrin-A usually binds to EphA receptors and Ephrin-B usually binds to EphB receptors. Eph-ephrin is involved in bi-directional signaling that regulates organ function, development of the nervous system, cell migration, axon guidance (Murai and Pasquale, 2003; Palmer and Klein, 2003), immunity, blood sugar balance and intestinal balance. Recently studies suggested that Eph receptor and its ligand Ephrin are involved with tumor formation and angiogenesis and their expression could affect prognosis (Wu et al., 2004; Jubb et al., 2005; Lugli et al., 2005;

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Guo et al., 2006; Fu et al., 2009; Senior et al., 2010). These findings make Eph of interest as tumor markers and new target genes for Gene Therapy. For this study, we analyzed the expression of EphB1/Ephrin-B in patients with Gallbladder Carcinoma, which has not been reported in Gallbladder Carcinoma.

## Materials and Methods

### Patient cohort

We collected 46 surgical specimens of gallbladder SC/ASC from Jan. 1995 to Dec. 2009. These tumors accounted for 4.34% (46/1060) of the Gallbladder Carcinoma cases diagnosed during that period. The specimens were collected from 7 hospitals. The average patient age was  $55.8 \pm 9.6$  years (mean + SD) (range: 35 -82). Detailed clinical pathological data are in Table 1. 80 surgical specimens of Gallbladder Carcinoma collected from Jan. 2000 to Dec. 2009 were examined for comparison (Table 1). This study was pre-approved by The Ethics Committee for Human Research, Second Xiangya Hospital, Central South University. The TNM classification of malignant tumor 7th edition, published by the International union against cancer (IUAC) was used for TNM staging of gallbladder cancer. Two year follow-up was obtained in all patients by letter or phone calls.

### Methods

Rabbit anti-human EphB1 and Ephrin-B polyclonal antibodies were purchased from Abgent Company (California, USA). The EnVision™ Detection Kit was purchased from Dako Laboratories (California, USA).

The EnVision detection kits were used for immunohistochemical staining of EphB1 and Ephrin-B. Kits were used per the manufacturer's instructions. The tissue sections were processed as follows. SC/ASC and AC specimens were fixed for 24 hours and cut into 4  $\mu$ m paraffin-embedded sections for routine use. Sections were dewaxed and washed with water  $\rightarrow$  the sections were placed into 3%  $H_2O_2$  in methanol for 10 min  $\rightarrow$  the specimens were digested with trypsin for 15 min  $\rightarrow$  one drop of rabbit anti-human EphB1 or Ephrin-B polyclonal antibody solution were placed on the specimen and incubated at 37°C for 30 min  $\rightarrow$  coloration was performed using developing solution for 15 min  $\rightarrow$  secondary staining was performed using hematoxylin for 1 min  $\rightarrow$  dehydration, placement of a cover slip and sealing with neutral gum. The cells with brown yellow particles within the cytoplasm were EphB1 and Ephrin-B positive cells. 400 cells were examined at 10 random fields. Greater than 25% positive cells were regarded as positive and less than 25% positive cells were regarded as negative. Positive sections provided by Beijing Zhongshan Golden Bridge Biotechnology Company (Beijing, China), Negative control was created by replacing the primary antibody with 5% PBS.

### Statistical analysis

Data was analyzed using SPSS13.0. The correlation between expression of EphB1 and Ephrin-B and

**Table 1. Comparison of Clinicopathological Characteristics, EphB1 and Ephrin-B Expression between SC/ASC and AC**

clinicopathological characteristic	SC/ASC (n=46)	AC (n=80)	$\chi^2$	P value
Sex				
male	19 (41.3)	26 (32.5)	0.986	0.352
female	27 (58.7)	54 (67.5)		
Age (year)				
$\leq 45$	3 (6.5)	16 (20.0)	4.143	0.042
$> 45$	43 (93.5)	64 (80.0)		
Differentiation				
well-differentiated	16 (34.8)	27 (33.8)		
moderately differentiated	24 (52.2)	25 (31.3)	8.515	0.014
poorly differentiated	6 (13.0)	28 (35.0)		
Maximum diameter of tumor (cm)				
$\leq 3$ cm	20 (43.5)	50 (62.5)	4.280	0.039
$> 3$ cm	26 (56.5)	30 (37.5)		
cholecystolithiasis				
-	18 (39.1)	42 (52.5)	2.093	0.148
+	28 (60.9)	38 (47.5)		
TNM stages				
I+II	12 (26.1)	21 (26.3)		
III	20 (33.5)	38 (47.5)	0.287	0.866
IV	14 (30.4)	21 (26.3)		
Lymph node metastasis				
-	17 (37.0)	30 (37.5)	0.004	0.952
+	29 (63.0)	50 (62.5)		
Locoregional invasion				
-	16 (34.8)	31 (38.8)	0.197	0.658
+	30 (62.5)	49 (61.3)		
Operation methods				
radical	14 (30.4)	26 (32.5)		
palliative	18 (39.1)	28 (35.0)	0.215	0.898
without resection	14 (30.4)	26 (32.5)		
mean survival time	10.07 (4-25)	10.34 (3-27)	0.014	0.906
EphB1				
-	27 (58.7)	45 (56.3)	0.000	0.995
+	19 (41.3)	35 (43.7)		
Ephrin-B				
-	25 (54.4)	46 (57.5)	0.196	0.658
+	21 (45.6)	34 (42.5)		

histological and clinical factors were examined using the  $\chi^2$  test and Fisher's exact test. Univariate survival analysis was performed using the Kaplan-Meier method and Log-Rank test. Multivariate analysis and confirmation of 95% confidence intervals were performed using the Cox proportional hazards model.

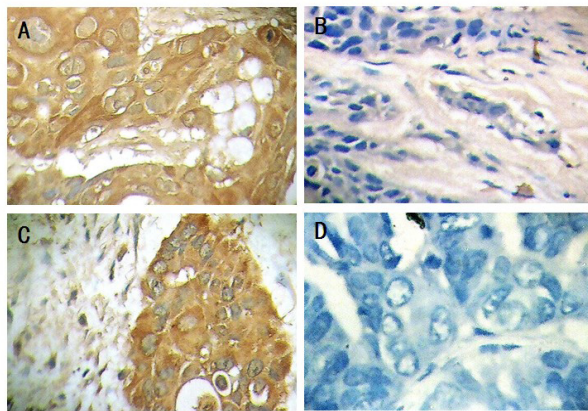
## Results

### Clinicopathological features of gallbladder SC/ASC and AC and expression of EphB1 and Ephrin-B

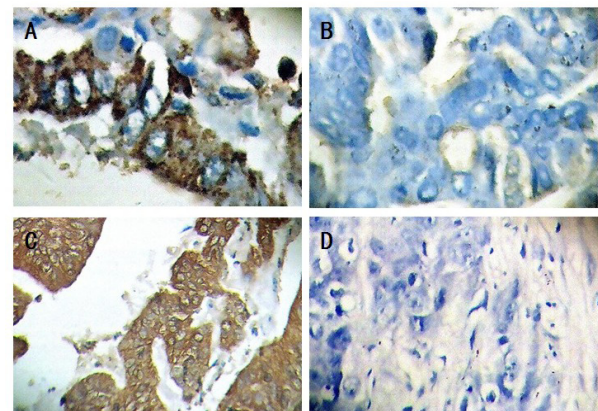
As shown in Table 1, SC/ASC tumors occurred more frequently in patients greater than 45 years of age and in tumors with a largest diameter  $> 3$ cm than AC tumors ( $P < 0.05$ ). The proportion of poorly differentiated adenocarcinoma in the AC group was higher than in the SC/ASC group ( $P < 0.05$ ). There were no significant differences in gender, frequency of gallbladder stones, TNM stage, frequency of lymph node metastases, local invasion, surgical approaches and average survival time in the SC/ASC and AC groups ( $P > 0.05$ ). Immunohistochemical staining for EphB1 and Ephrin-B was visualized mainly in the cytoplasm, with occasional staining seen in the nucleus (Figure 1 and

**Table 2. The Association of EphB1 and Ephrin-B Expression with the Clinicopathological Characteristics of SC/ASC**

clinicopathological characteristic	Total No:	EphB1			Ephrin-B		
		Pos No: (%)	$\chi^2$	P	Pos No: (%)	$\chi^2$	P
<b>Histopathologic subtypes*</b>							
SC	26	10 (38.5)	0.199	0.655	11 (42.3)	0.270	0.604
ASC	20	9 (45.0)			10 (50.0)		
<b>Differentiation</b>							
well-differentiated	16	8 (50.0)	2.003	0.367	11 (68.8)	6.115	0.047
moderately differentiated	24	10 (41.7)			9 (37.5)		
poorly differentiated	6	1 (16.7)			5 (83.3)		
<b>Maximum diameter of tumor (cm)</b>							
≤3cm	20	12 (60.0)	5.101	0.024	14 (70.0)	8.455	0.004
>3cm	26	7 (26.9)			7 (26.9)		
<b>Cholecystolithiasis</b>							
-	18	7 (38.9)	0.071	0.790	6 (33.3)	1.809	0.179
+	28	12 (42.9)			15 (53.6)		
<b>TNM stages</b>							
I+II	12	9 (75.0)	9.844	0.021	9 (75.0)	6.072	0.050
III	20	8 (40.0)			8 (40.0)		
IV	14	2 (14.3)			4 (28.6)		
<b>Lymph node metastasis</b>							
-	17	11 (64.7)	6.091	0.014	13 (76.5)	10.323	0.001
+	29	8 (27.6)			8 (27.6)		
<b>Locoregional invasion</b>							
-	16	12 (75.0)	11.49	0.001	11 (68.8)	5.275	0.024
+	30	7 (23.3)			10 (33.3)		
<b>Operation methods</b>							
radical	14	10 (71.4)	7.993	0.018	10 (71.4)	7.070	0.028
palliative	18	6 (33.3)			8 (44.4)		
without resection	14	3 (21.4)			3 (21.4)		



**Figure 1. EphB1 and Ephrin-B Expression in SC/ASC.** EnVision immunohistochemistry, original magnification x200. EphB1 and Ephrin-B positive reaction was mainly localized in the cytoplasm. A, Positive EphB1 expression in well differentiated SC/ASC. B, Negative EphB1 expression in poorly differentiated SC/ASC. C, Positive Ephrin-B expression in moderately differentiated SC/ASC. D, Negative Ephrin-B expression in poorly differentiated gallbladder SC/ASC



**Figure 2. EphB1 and Ephrin-B Expression in AC.** EnVision immunohistochemistry, original magnification x200. EphB1 and Ephrin-B positive reaction was mainly localized in the cytoplasm. A, Positive EphB1 expression in well-differentiated AC. B, Negative EphB1 expression in moderately differentiated AC. C, Positive Ephrin-B expression in well-differentiated AC. D, Negative Ephrin-B expression in poorly differentiated AC

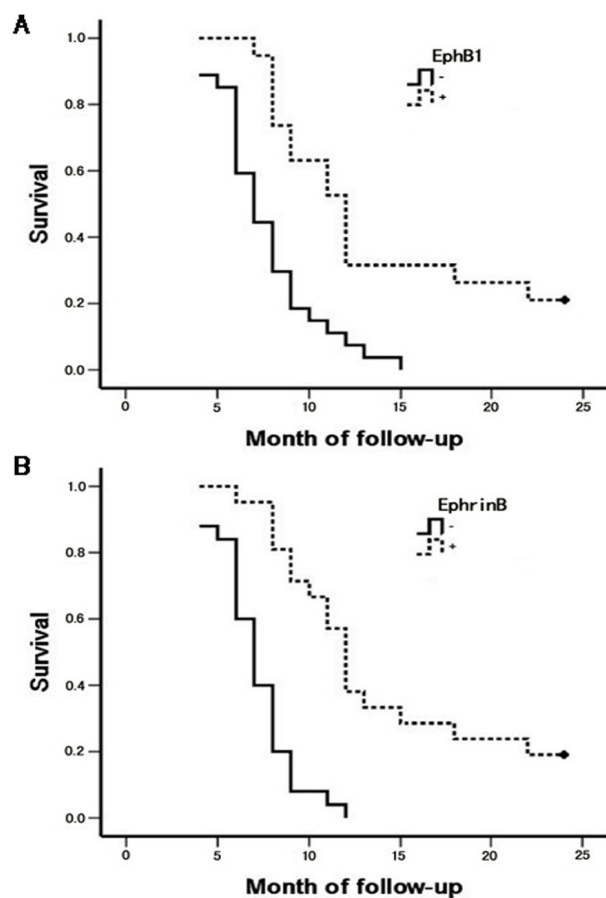
Figure 2). Among the 46 SC/ASC cases, 19 (41.3%) were EphB1 positive and 21 (45.6%) were Ephrin-B positive. ASC tumors with a positive adenocarcinoma component and a negative SC component were regarded as negative. Among the 80 AC cases, positive expression for EphB1 and Ephrin-B was seen in 43.7% (35 cases) and 42.5% (34 cases) of tumors, respectively. There was no significant difference in positive expression of EphB1 and Ephrin-B in the SC/ASC and AC tumors ( $P>0.05$ ).

#### Correlation between expression of EphB1 and Ephrin-B and clinical pathological features of patients with SC/ASC or AC

As shown in Table 2, expression rate of EphB1 and Ephrin-B was higher in SC/ASC with tumors measuring  $\leq 3$ cm, stagel and stage II (TNM stage) tumors, tumor from patients with no lymph node metastasis, no invasion and in tumors removed by radical resection ( $p<0.05$  or  $p<0.01$ ). The expression of Ephrin-B in well

**Table 3. The Association of EphB1 and Ephrin-B Expression with the Clinicopathological Characteristics of AC**

clinicopathological characteristic	Total No:	EphB1			Ephrin-B		
		Pos No: (%)	$\chi^2$	P	Pos No: (%)	$\chi^2$	P
Differentiation							
well-differentiated	27	17 (63.0)	12.37	0.002	16 (59.3)	6.64	0.038
moderately differentiated	25	13 (52.0)			11 (44.0)		
poorly differentiated	28	5 (17.9)			7 (25.0)		
Maximum diameter of tumor (cm)							
$\leq 3$ cm	50	28 (56.0)	8.130	0.004	26 (52.0)	11.002	0.001
$> 3$ cm	30	7 (23.3)			8 (26.7)		
Cholecystolithiasis							
-	42	21 (50.0)	1.404	0.236	19 (45.2)	0.271	0.602
+	38	14 (36.8)			15 (39.5)		
TNM stages							
I+II	21	15 (71.4)	11.764	0.003	14 (66.7)	9.744	0.008
III	38	16 (42.1)			16 (42.1)		
IV	21	4 (19.1)			4 (19.1)		
Lymph node metastasis							
-	30	19 (63.3)	7.480	0.005	18 (60.0)	6.015	0.014
+	50	16 (32.0)			16 (32.0)		
Locoregional invasion							
-	31	21 (67.7)	11.838	0.001	21 (67.7)	13.196	0.000
+	49	14 (28.6)			13 (26.5)		
Operation methods							
radical	26	19 (73.1)	17.596	0.000	17 (65.4)	11.334	0.003
palliative	28	12 (42.9)			12 (42.9)		
without resection	26	4 (15.4)			5 (19.2)		

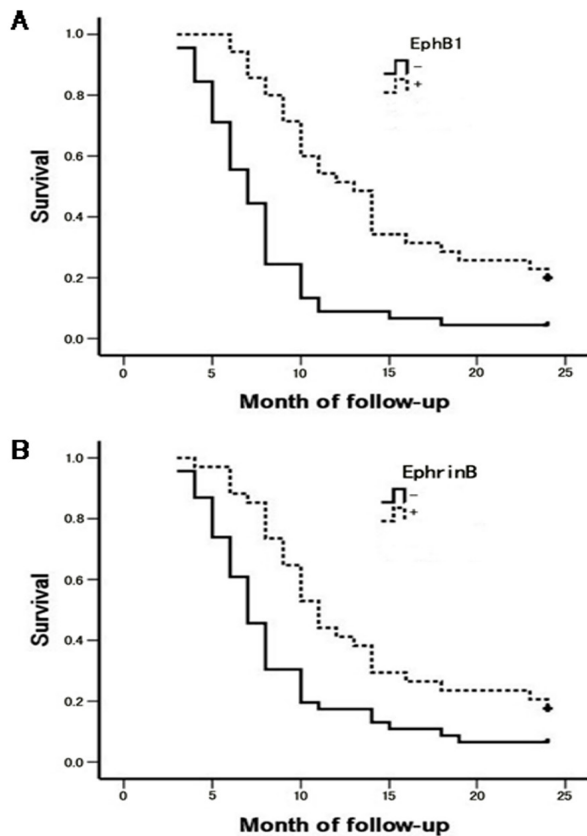


**Figure 3. EphB1 and Ephrin-B Expression and Survival in Patients with SC/ASC.** A, Kaplan-Meier plots of overall survival in patients with SC/ASC and with EphB1 positive and negative expression. B, Kaplan-Meier plots of overall survival in patients with SC/ASC and with Ephrin-B positive and negative expression

differentiated SC/ASC tumors was significantly higher than in poorly differentiated tumors ( $P < 0.05$ ). Our observation implicated that there was no difference in expression by gender, age, pathological type or presence of gallstones ( $P > 0.05$ ) (Table 2). As shown in Table 3, expression of EphB1 and Ephrin-B was higher in cases of AC with well differentiation, small tumor mass, lower TNM stage with no lymph node metastasis, no invasion into the gallbladder surrounding tissues and organs ( $p < 0.05$  or  $p < 0.01$ ). There was no correlation of expression with other features. ( $P > 0.05$ ).

*The association between clinical pathological parameters, expression of EphB1 and Ephrin-B and average survival of SC/ASC and AC patients*

We obtained follow-up data from all 46 SC/ASC cases by letter or phone calls. Only 13 of the 46 SC/ASC patients survived more than 1 year, and 4 patients survived longer than 2 years. The average survival was  $10.1 \pm 0.78$  months. Kaplan-Meier survival analysis revealed that degree of differentiation, tumor diameter, TNM stage, lymph node metastases, invasion of surrounding organs, surgical approaches and average patient age were closely related to average survival. Patients with tumors that were positive for EphB1 and Ephrin-B had significantly longer survival time than those with negative expression ( $P < 0.05$  or  $P < 0.01$ ) (Figure 3). Cox multivariate analysis indicated poor prognosis for survival for poorly differentiated tumors, larger tumor diameter  $> 3$ cm, stage IV disease, positive lymph node metastases, local invasion of adjacent organs, and less than radical resection. Cox multivariate analysis also indicated that tumors expressing EphB1 and Ephrin-B have a better overall survival, suggesting that they are strong independent protected factors of SC/ASC



**Figure 4. EphB1 and Ephrin-B Expression and Survival in Patients with AC.** A, Kaplan-Meier plots of overall survival in patients with AC and with EphB1 positive and negative expression. B, Kaplan-Meier plots of overall survival in patients with AC and with Ephrin-B positive and negative expression

( $P < 0.05$ ).

We found 23 of the 80 AC patients survived longer than 1 year and 9 patients survived greater than 2 years. The average survival was  $10.3 \pm 0.63$  months. The Kaplan-Meier survival analysis and Cox multivariate analysis of AC patients revealed similar results as SC/ASC patients. Patients with tumors that were positive for EphB1 and Ephrin-B had significantly longer survival than those with negative expression ( $P < 0.01$ ) (Figure 4). However, expression of EphB1 and Ephrin-B predicted for better survival, both of them are independent protected factors of AC ( $P < 0.05$ ).

## Discussion

There are few clinicopathologic reports of SC/ASC of the gallbladder, but none to our knowledge have evaluated the clinicopathologic features of the SC/ASC along with the AC in relation to Eph receptors, important proteins related to oncogenesis. In this study we identified 80 gallbladder AC and 46 gallbladder SC/ASC. The collected 46 SC/ASC cases accounted for 4.34% of the gallbladder cancers, similar to previous reports. The proportion of SC/ASC tumors with a diameter larger than 3cm (56.5%) was significantly higher than that in AC (37.5%), but there was no difference in the rate of lymph node metastases or local invasion. Although women were more commonly affected in the literature, we found no gender difference

in SC/ASC or AC patients. The radical resection rate for the 46 SC/ASC cases was 30.4%, similar to that for AC. Patient survival was significantly higher with radical resection than with palliative or no resection. SC/ASC and AC had similar clinical manifestations, biological behavior, treatment approach and prognosis. Additionally, we found that EphB1 and Ephrin-B overexpression were significantly associated with small tumor size, low TNM stage, no metastasis and invasion and good prognosis of AC and SC/ASC patients.

Abnormal expression of Eph/Ephrin has been reported in many tumors and can also act as both tumor promoters and suppressors in different contexts (Chen et al., 2008; Genander et al., 2009; Miao et al., 2009; Mao et al., 2013). Early studies suggested the Eph family were oncogenes that can enhance tumor occurrence and development. In 2004 and 2005, EphB2 expression has been found to be promoting glioma cell invasion, suggesting EphB2 acts like a tumor -promoting activities (Nakada et al., 2004; Nakada et al., 2005). Conversely, Batlle E and Davalos V reported expression of EphB2 and EphB4 inhibited tumorigenesis, and might be used as a targeting gene for treatment of colorectal cancer (Batlle et al., 2005; Davalos et al., 2006). These divergent and paradoxical Eph and Ephrin signaling functions make it of great interest to study the role of Eph and Ephrin in AC and SC/ASC. In this study, we found Ephrin-B and Ephrin-B expression in AC and SC/ASC to be associated with well differentiated tumors, tumors less than 3 cm in diameter, stage I+II cancers, absence of lymph node metastases, absence of local invasion and radically resectable tumors. Importantly, we found a positive correlation between EphB1 and EphrinB1 expression and better survival on multivariate Cox regression analysis. However, it had been suggested that EphA2 could enhance the development of gastric carcinoma by reducing Epithelial-Mesenchymal Transition (EMT) of cancer cells by activating WNT/ $\beta$ -catenin signaling (Huang et al., 2013), which was conflicted to our study. Therefore, the expression of Eph/Ephrin and their role in tumor progression seems to be tumor type specific. In our study, expression of Ephrin-B and Ephrin-B may reflect the progression and clinical behavior of GBC. We believe EphB1 and EphrinB1 should be used as molecular markers for early diagnosis and estimating the prognosis of gallbladder AC and SC/ASC.

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