

Expression levels of *EPHB4*, *EFNB2* and caspase-8 are associated with clinicopathological features and progression of esophageal squamous cell cancer

QIANZHI NI^{1,2}, PINGPING CHEN³, BING ZHU², JINGJING LI², DONG XIE^{2,4} and XINGYUAN MA¹

¹School of Biotechnology, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237; ²CAS Key Laboratory of Nutrition, Metabolism and Food Safety, Shanghai Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences, University of the Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031; ³Department of Health Statistics, College of Public Health, Zhengzhou University, Zhengzhou, Henan 45001; ⁴NHC Key Laboratory of Food Safety Risk Assessment, China National Center for Food Safety Risk Assessment, Beijing 100022, P.R. China

Received May 28, 2019; Accepted October 16, 2019

DOI: 10.3892/ol.2019.11160

Abstract. The upregulation of EPH receptor B4 (*EPHB4*) results in a survival advantage for tumor cells via the inhibition of the caspase-8-mediated apoptotic pathway, which begins from the cell membrane. The present study investigated the expression patterns of *EPHB4*, ephrin B2 (*EFNB2*) and caspase-8 in patients with esophageal squamous cell carcinoma (ESCC). The association between the expression patterns and certain clinicopathological characteristics of the patients was also determined. mRNA levels of *EPHB4*, *EFNB2* and caspase-8 in paired primary ESCC samples and adjacent esophageal tissues collected from 96 patients with ESCC were quantified using quantitative PCR. Upregulation of *EPHB4* and *EFNB2* mRNA expression, and downregulation of caspase-8 mRNA were detected in ESCC samples compared with that in the adjacent esophageal tissues. The expression levels of *EPHB4* and *EFNB2* were positively correlated with each other, whereas the mRNA levels of both *EPHB4* and *EFNB2* exhibited a negative correlation with that of caspase-8. The mRNA levels of both *EPHB4* and *EFNB2* demonstrated a significant positive association with certain

clinicopathological features of patients with ESCC, including family history, tumor size, metastasis and stage. Conversely, a negative association was revealed between the expression level of caspase-8 and clinicopathological features of patients with ESCC. Moreover, mRNA expression levels of *EPHB4* and *EFNB2* were negatively associated with survival times of patients with ESCC, whereas the level of caspase-8 was positively associated with patient outcome. The results from the present study suggested that *EPHB4*, *EFNB2* and caspase-8 may be implicated in the tumorigenesis and progression of ESCC, and that consequently, they may serve as useful prognostic markers, as well as potential therapeutic targets.

Introduction

The Eph receptor family comprises the largest receptor tyrosine kinase superfamily, and contains 14 distinct members, with 9 molecules identified in its ligand ephrin family (1). According to their sequence homology and binding specificity, both Ephs and ephrins are classified as type A and B. Upon engagement of Eph by the cognate ephrin, the two molecules activate simultaneously and induce intracellular signal transduction, which initiates a number of biological processes, including axon guidance, neural crest cell migration, hindbrain segmentation, somite formation and vasculogenesis (2,3). Moreover, there is accumulating evidence that the Eph/ephrin system also serves a pivotal role in the development and progression of numerous cancer types (4,5).

EPH receptor A2 (*EphA2*) is the most well characterized of the Eph receptors, particularly when regarding its role in tumorigenesis; it has been revealed to be upregulated in a number of different types of tumor, including prostate, colon and lung cancer, as well as melanomas (4). Furthermore, overexpression of *EphA2* is able to induce malignant transformation in mammary epithelial cells (4). The EphB/ephrinB system is also implicated in tumorigenesis (4). The expression level of *EphB2* is reported to be upregulated in gastrointestinal, liver, ovarian, lung and renal cancers (4). Although the majority of

Correspondence to: Professor Xingyuan Ma, School of Biotechnology, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, P.R. China
E-mail: xingyuanma03@gmail.com

Professor Dong Xie, CAS Key Laboratory of Nutrition, Metabolism and Food Safety, Shanghai Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences, University of the Chinese Academy of Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, P.R. China
E-mail: dxie@sibs.ac.cn

Key words: *EPHB4*, *EFNB2*, caspase-8, esophageal squamous cell cancer

studies suggest that Ephs and ephrins serve an oncogenic role, *EphB2* was reported as a tumor suppressor in prostate and colorectal tumors (6-8). These findings reflect the complexity of the differential functions of the Eph/ephrin system, which is capable of exerting context-dependent agonistic or antagonistic effects. Caspase-8, a member of the cysteine-aspartic acid protease (caspase) family, is well characterized as an initiator of death receptor-mediated apoptosis, and has been implicated in other similar apoptotic responses (9). Caspase-8 promoter methylation results in the loss of gene expression, which is associated with tumor severity in a variety of different tumor types. The methylation-mediated silencing of key apoptosis-associated genes serves an important role in the pathogenesis and development of therapeutic resistance in human cancer cells (10).

Esophageal cancer represents the sixth most frequent cause of cancer-associated mortality worldwide (11). Esophageal squamous cell carcinoma (ESCC) is the most prevalent histological subtype of esophageal cancer and exhibits high mortality rates and a 5-year overall survival rate of $\leq 15\%$ (12,13). The most common pathological subtypes of esophageal cancer are ESCC and esophageal adenocarcinoma. Despite the well-characterized pathological progression of ESCC, the underlying molecular mechanisms are predominantly yet to be elucidated. Several studies reported that the expression of *EphA2* (and one of its receptors, ephrinA1) were upregulated in ESCC, and correlated with tumor progression and patient survival, revealing their predictive potential for the diagnosis and prognosis of patients with ESCC (14). Previous studies demonstrated that *EPHB4* conferred a survival advantage on tumor cells by decreasing apoptosis, whereas knockdown of *EPHB4* expression using siRNA induced apoptosis and decreased tumor cell viability via the activation of caspase-8. However, studies focusing on the influence that EphB/ephrin-B and caspase-8 exert on ESCC progression and genesis remain limited. Therefore, the present study investigated the expression levels of *EPHB4*, its cognate ligand ephrin B2 (*EFNB2*) (1-3) and caspase-8 in ESCC. In addition, the association between their relative expression levels and clinical parameters important in the diagnosis and prognosis of ESCC were also investigated in the present study. The results from the present study provide additional understanding, potentially facilitating the development of diagnostic and therapeutic strategies for the treatment of ESCC.

Materials and methods

Patients and samples. In the present study, 96 ESCC samples, and their paired paracancerous esophageal tissues, were obtained from patients with ESCC treated at the First Affiliated Hospital of Zhengzhou University (Henan, China), between July 2002 and August 2006, following the provision of written informed consent. The tumor stage was classified according to the 8th edition of the TNM classification (15). Cancerous tissues were surgically resected from patients who had not received any neo-adjuvant therapy, and the corresponding non-cancerous 'normal' tissues, located at least 3 cm away from the tumor site, were obtained in the same manner. Each specimen was divided into 2 pieces, one of which was fixed in 4% formalin at 4°C overnight, sectioned and examined

using immunohistochemical (IHC) staining, and the other of which was stored at -80°C. The present study was approved by the Institutional Review Board of the Institute for Nutritional Sciences, Chinese Academy of Sciences.

Quantitative (q)PCR. RNA extraction, DNA template synthesis and amplification reactions were performed as previously described (16). The primers for the qPCR are listed as follows: *EPHB4* forward, 5'-TCCTTCCTGCGGCTAAC-3' and reverse, 5'-CTTTGCAGACGAGGTTGCT-3'; *EFNB2* forward, 5'-TCTTTGGAGGGCCTGGATAA-3' and reverse, 5'-CGTCTGTGCTAGAACCTGGATT-3'; caspase-8 forward, 5'-CTGCAGAGGAACCTGGTACATCC-3' and reverse, 5'-TCTTACTCCAAGGTGGCCATG-3'; and β -actin forward, 5'-GATCATGTGCTCCTCCTGAGC-3' and reverse, 5'-ACTCCTGCTTGCTGATCCAC-3'. All primers were designed using PRIMER5 software (version 5.00; Premier Biosoft International) and purchased from Shanghai Sangong Pharmaceutical Co., Ltd. Reactions were characterized at the point during cycling when amplification of the PCR product was first detected after a fixed number of cycles. Quantification was performed by measuring the quantitation cycle (Cq) value. The levels of target genes in each sample were normalized to the housekeeping gene β -actin via the following formula: Normalized level (NL) = $\text{level}_{(\text{target})} / \text{level}_{(\beta\text{-actin})} = 2^{\text{Cq}(\text{target}) - \text{Cq}(\beta\text{-actin})} = 2^{\Delta\text{Cq}}$. Furthermore, the relative levels (RL) of target genes in cancer tissues vs. corresponding normal samples were calculated according to the formula: $\text{RL} = \text{NL}_{(\text{cancer})} / \text{NL}_{(\text{normal})} = 2^{\Delta\text{Cq}(\text{cancer}) - \Delta\text{Cq}(\text{normal})} = 2^{\Delta\Delta\text{Cq}}$. As both NL and RL are represented as 2^{Cq} , the present study used ΔCq and $\Delta\Delta\text{Cq}$ to represent NL and RL, respectively, when performing statistical analysis.

IHC. The specimens were fixed in 4% formalin at 4°C overnight. The paraffin-embedded tissues were cut into 5- μm thick sections, deparaffinized, rehydrated in graded dimethylbenzene and ethanol solutions, and subjected to antigen retrieval. Subsequently, the sections were blocked using 5% normal goat serum (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. The tissue sections were then incubated with the following primary antibodies at 4°C overnight: Rabbit anti-human *EPHB4* (1:500; cat. no. sc-365510), rabbit anti-human *EFNB2* (1:500, cat. no. sc-398735) (both Santa Cruz Biotechnology, Inc.) and rabbit anti-human caspase-8 (1:100; cat. no. 552143; BD Pharmingen; BD Biosciences). Following primary incubation, the sections were incubated at 37°C for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1,000; cat. no. A-10194; Chemicon International; Thermo Fisher Scientific Inc.). Finally, all sections were counterstained with hematoxylin at room temperature for 5-8 min.

Scoring of IHC staining was simultaneously performed by three independent pathologists. Tumor cells positive and negative for staining were counted separately under a light microscope (magnification, $\times 200$). For each slide, 7-10 microscopic fields with ≥ 300 cells/microscopic field were randomly selected. The ratio of positive cells was calculated as the number of positively stained tumor cells divided by the total tumor cells, in each high-power field area. The level of protein expression was quantified by calculating the percentage ratio

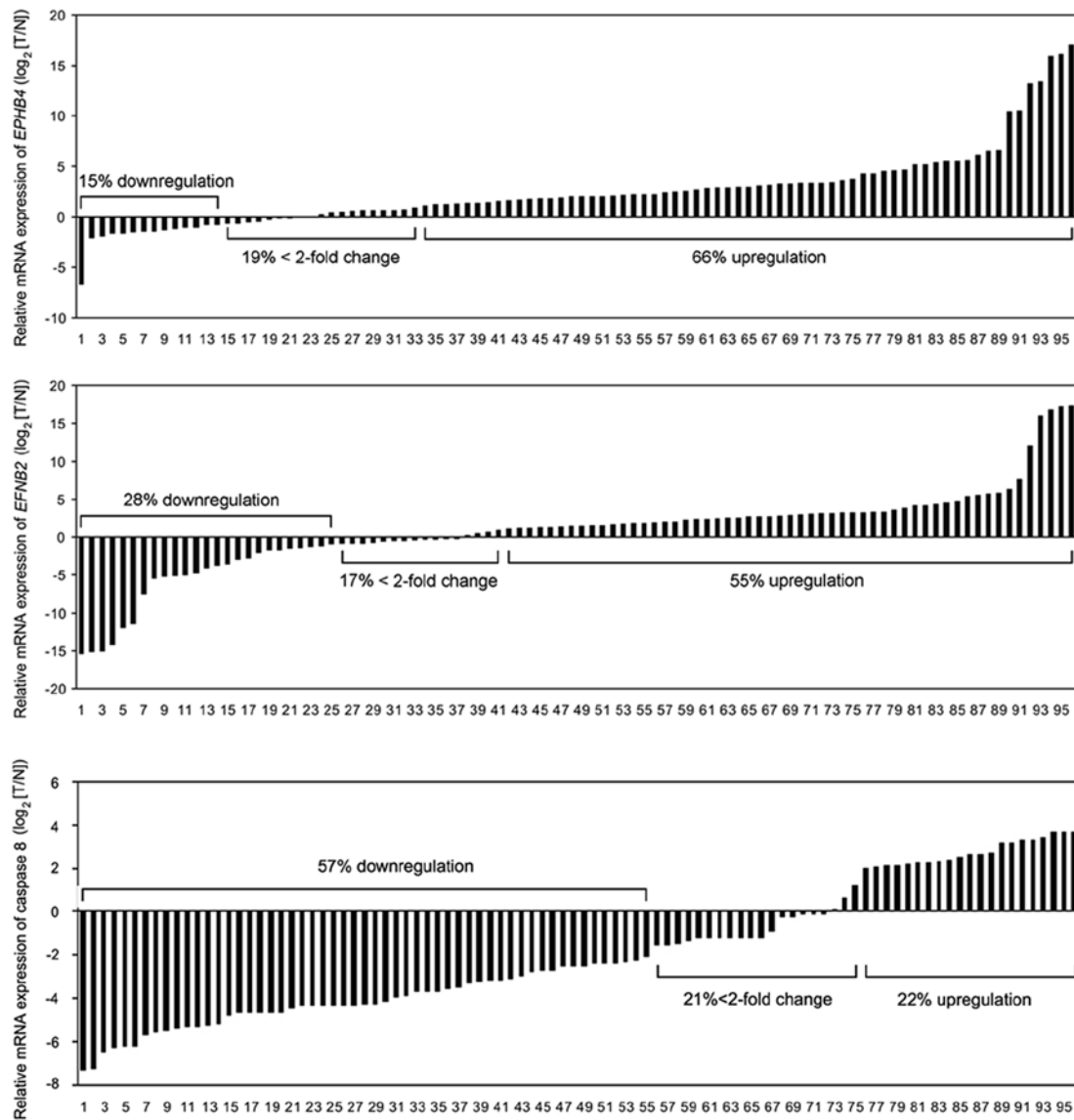


Figure 1. Expression patterns of *EPHB4* and *EFNB2* in ESCC compared with those in matched normal esophageal tissues. Relative mRNA expression levels of *EPHB4* and *EFNB2* in human ESCC and paired paracancerous esophageal tissues were examined using quantitative PCR. Each bar is the log₂ value of the ratio of either *EPHB4* or *EFNB2* mRNA level between (T) ESCC and (N) paired paracancerous tissues from the same patient. Less than 2-fold change: The ratio between tumor and normal tissue is <2. Moreover, as Log₂=1, bar value >1 represents >2-fold increase (T > N), whereas bar value <-1 represents >2-fold decrease (T < N). *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2; ESCC, esophageal squamous cell carcinoma.

of positively stained cells in the esophageal cancer sample compared with that in the matched paracancerous esophageal tissues. Patients with high expression of *EPHB4*, *EFNB2* and caspase-8 had protein levels of ≥ 1.89 , ≥ 1.57 and ≥ 0.56 , respectively; whereas patients with low expression had protein levels of < 1.89 , < 1.57 and < 0.56 , respectively.

Statistical analysis. The χ^2 test was used to determine the association between the expression levels of *EPHB4*, *EFNB2* and caspase-8 in ESCC samples and the clinical characteristics, respectively. Pearson's correlation analysis was used to estimate the relative degree. Kaplan-Meier survival curves comparing patients with high and low expression at the mRNA and protein levels were plotted and univariate survival analysis was performed using log-rank test.

Multivariate analyses were performed to estimate the effects of certain clinicopathological characteristics, and the

expression levels of the two genes, on survival. The data were analyzed using Student's t-test. $P < 0.05$ were considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 22 (IBM Corporation).

Results

Expression of *EPHB4*, *EFNB2* and caspase-8 genes in ESCC and matched normal esophageal tissues. In order to investigate the expression pattern of *EPHB4*, *EFNB2* and caspase-8 in ESCC, the mRNA levels of the three genes were quantified in 96 pairs of tumor samples and matched normal esophageal tissue samples using qPCR. Expression levels were presented as a ratio between *EPHB4*, *EFNB2* or caspase-8 and the reference gene β -actin. Upregulation of *EPHB4* and *EFNB2* occurred in 63 out of 96 (66%) ESCC samples, and 53 out of 96 (55%) paired normal esophageal tissues, respectively.

Table I. Expression of *EPHB4*, *EFNB2* and caspase-8 genes in esophageal cancer and paired paracancerous esophageal tissues (n=96 pairs).

| mRNA/protein | n | Cancerous | Matched-paracancerous | (N-C)/(C/N) | t-test | P-value |
|----------------------|----|-------------|-----------------------|-------------|---------|---------------------|
| <i>EPHB4</i> mRNA | 96 | 12.89±10.08 | 15.45±10.26 | 2.56±3.92 | 6.411 | <0.01 ^a |
| <i>EFNB2</i> mRNA | 96 | 8.05±5.88 | 8.86±5.69 | 0.81±5.77 | 1.375 | 0.172 |
| caspase-8 mRNA | 96 | 7.38±2.47 | 5.46±1.87 | -1.92±2.67 | 7.307 | <0.001 ^a |
| <i>EPHB4</i> protein | 96 | 21.35±8.296 | 2.80±0.947 | 8.74±5.65 | -21.603 | <0.001 ^a |
| <i>EFNB2</i> protein | 96 | 11.67±2.478 | 1.71±0.597 | 7.83±3.44 | -38.322 | <0.001 ^a |
| Caspase-8 protein | 96 | 2.51±2.384 | 8.85±7.879 | 0.28±0.15 | -22.761 | <0.001 ^a |

^aP<0.05. N-C, Cq value of normal esophageal minus that of cancerous esophageal tissues; C/N, percentage of positively stained cells in esophageal cancer tissues compared with the matched normal samples; *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2.

By contrast, downregulation of caspase-8 was observed in 55 out of 96 (57%) ESCC samples, when compared with that in normal tissues (Fig. 1). Univariate analysis revealed that the mRNA level of *EPHB4* was significantly increased in tumor tissues, compared with paired normal tissues (P=0.001), while the expression level of caspase-8 was significantly lower in the ESCC samples (P=0.001). However, there was no significant difference in the mRNA level of *EFNB2* between the ESCC and paired normal tissues (P=0.172) (Table I). Pearson's correlation analysis demonstrated that the mRNA expression of *EPHB4* was positively correlated with that of *EFNB2* ($R^2=0.620$; P<0.001). Notably, *EPHB4* ($R^2=-0.428$; P=0.001) and *EFNB2* ($R^2=-0.267$, P=0.028) were both negatively correlated with caspase-8 (Table II).

Subsequently, IHC was performed to investigate the protein expression levels of *EPHB4*, *EFNB2* and caspase-8 proteins in 96 pairs of esophageal tissues. As presented in Fig. 2A and C, *EPHB4* and *EFNB2* proteins were not apparent in the majority of normal esophageal epithelial cells, while they were highly expressed in the majority tumor cells in corresponding ESCC tissues (Fig. 2B and D), synonymous with previous reports (14,17). As presented in Fig. 2E, strong staining of caspase-8 was observed in the superficial layer of normal esophageal epithelia, but was almost undetectable in the ESCC samples (Fig. 2F), which is also consistent with other studies (18,19). The IHC scoring analysis revealed that the ratio of *EPHB4*/*EFNB2*-positive to -negative cells in ESCC tissues was significantly higher in comparison with that in the corresponding normal tissue, whereas the ratio of caspase-8-positive to -negative cells was lower in ESCC tissues compared with that in their normal counterparts (Table I).

Association between the expression of *EPHB4*, *EFNB2* and caspase-8, and the clinicopathological features of patients with ESCC. The univariate analysis revealed a significant association between the expression of *EPHB4* and family history, metastasis, and tumor size, position and stage. The expression level of *EPHB4* was significantly higher in patients with a family history of cancer (P<0.001). A significant association also existed between increased levels of *EPHB4* and metastasis (P=0.001), larger tumors (P=0.001), ESCC located in the lower segment of the esophagus (P=0.010) and a higher stage (P=0.043), indicating that the upregulation of *EPHB4*

Table II. Pearson's correlation analysis of mRNA and protein expression of *EPHB4*, *EFNB2* and caspase-8 in esophageal cancer and matched normal esophageal tissues (n=96 pairs).

| Genes | mRNA | | Protein | |
|-------------------------------|---------|---------------------|---------|--------------------|
| | R-value | P-value | R-value | P-value |
| <i>EPHB4</i> and <i>EFNB2</i> | 0.620 | <0.001 ^a | 0.202 | 0.049 ^a |
| <i>EPHB4</i> and caspase-8 | -0.428 | <0.001 ^a | -0.340 | 0.001 ^a |
| <i>EFNB2</i> and caspase-8 | -0.267 | 0.028 ^a | -0.198 | 0.041 ^a |

^aP<0.05. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2.

expression was associated with ESCC progression. Sex and age were not significantly associated with the expression level of *EPHB4* (Table III).

Statistical analysis also demonstrated that the expression level of *EFNB2* was significantly associated with several clinical features, including tumor position and family history. The mRNA level of *EFNB2* was significantly higher in the patients with a family history of cancer (P<0.001). ESCCs located in the lower segment of the esophagus exhibited higher *EFNB2* expression than those in the upper segment (P=0.048). However, no associations were observed between the expression level of *EFNB2* and sex, age, metastasis or tumor size and stage (Table III).

The expression level of caspase-8 was significantly downregulated in patients with family history (P=0.012). Downregulated expression levels of caspase-8 were significantly associated with metastasis (P<0.000), increased tumor size (P<0.000), ESCC at the lower segment of the esophagus (P=0.019) and a higher stage (P<0.000), indicating that low caspase-8 expression is associated with the progression of ESCC (Table III). However, there was no significant association observed between caspase-8 expression and sex or age.

IHC scoring analysis revealed that the ratio of *EPHB4*-positive to -negative cells in tissue samples was higher in patients with a family history of cancer (P=0.002). There was also a significant association between a higher positive-staining ratio and metastasis (P=0.005), larger

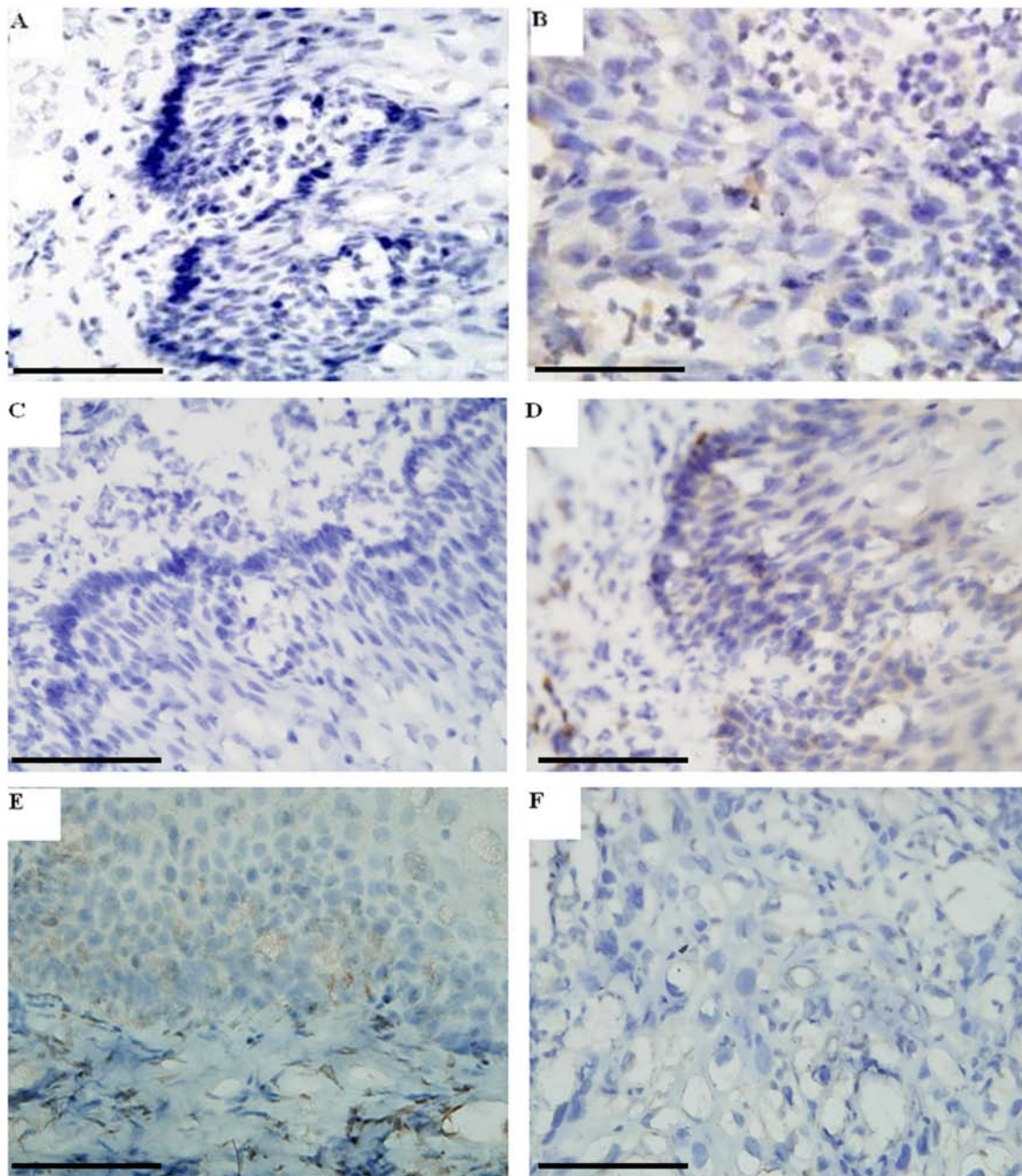


Figure 2. Representative immunohistochemical staining for *EPHB4*, *EFNB2* and caspase-8 proteins in ESCC and matched paracancerous esophageal tissues. (A) *EPHB4* and (C) *EFNB2* were observed in only a few cells in the normal esophageal tissues. (B) *EPHB4* and (D) *EFNB2* were expressed abundantly in ESCC tissues. By contrast, the expression of caspase-8 was observable in (E) paracancerous esophageal tissues, whilst undetectable in (F) ESCC tissues. All sections were counterstained using hematoxylin. Scale bar, 100 μm . *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2.

tumors ($P < 0.001$) and higher tumor stages ($P = 0.004$). The increased ratio of *EFNB2*-positive to -negative cells, as well as a decreased ratio of caspase-8 was identified in patients with metastasis or greater tumors, respectively (*EFNB2*, $P = 0.004$ and $P = 0.018$, respectively; and caspase-8, $P = 0.000$ and $P = 0.000$, respectively) (Table IV). Taken together, the associations between protein levels of *EPHB4*, *EFNB2* or caspase-8 and certain clinicopathological features of patients with ESCC (according to IHC), were consistent with the results concerning the mRNA levels.

Expression of EphB4, EFNB2 or caspase-8 and clinical outcomes of ESCC. The univariate survival analysis demon-

strated that patient age, family history and tumor metastasis were all significantly associated with survival time. The mRNA expression levels of *EPHB4* and *EFNB2* but not caspase-8, was associated with survival time and the protein expression levels of *EPHB4* and caspase-8, but not *EFNB2*, was associated with survival time (Table V). Kaplan-Meier curves indicated that patients with higher mRNA ($P < 0.0001$) and protein ($P < 0.0001$) expression levels of *EPHB4* exhibited a significantly shortened median survival time, compared with patients with lower expression levels (Fig. 3A and B; Table V). Similarly, patients with higher mRNA level of *EFNB2* expression ($P = 0.041$; Fig. 3C), or patients with lower protein level expression of caspase-8 expression ($P = 0.045$; Fig. 3F) also

Table III. Association between the levels of *EPHB4*/*EFNB2* and caspase-8 mRNA expression and the clinical and pathological features of individuals with esophageal cancer (n=96 pairs).

| Factors | <i>EPHB4</i> | | | <i>EFNB2</i> | | | Caspase-8 | | |
|-----------------------------|--------------|--------|----------|---------------------|---------|--------|-----------|--------|---------------------|
| | High, n | Low, n | χ^2 | P-value | High, n | Low, n | High, n | Low, n | P-value |
| Sex | | | | | | | | | |
| Male | 45 | 22 | 0.233 | 0.629 | 36 | 31 | 26 | 41 | 0.526 |
| Female | 18 | 11 | | | 19 | 10 | 15 | 14 | |
| Age, years | | | | | | | | | |
| ≤35 | 2 | 0 | 2.433 | 0.303 | 2 | 0 | 1 | 1 | 1.733 |
| 35-50 | 12 | 10 | | | 11 | 11 | 12 | 10 | |
| ≥50 | 49 | 23 | | | 42 | 30 | 28 | 44 | 0.494 |
| Metastasis | | | | | | | | | |
| Yes | 45 | 12 | 11.039 | 0.001 ^a | 20 | 19 | 9 | 48 | 41.552 |
| No | 18 | 21 | | | 35 | 22 | 32 | 7 | <0.001 ^a |
| Family history | | | | | | | | | |
| Yes | 53 | 2 | 53.940 | <0.001 ^a | 41 | 14 | 17 | 38 | 7.327 |
| No | 10 | 31 | | | 14 | 27 | 24 | 17 | 0.012 ^a |
| Tumor size, cm ³ | | | | | | | | | |
| ≤100 | 14 | 20 | 14.415 | 0.001 ^a | 15 | 19 | 27 | 7 | 32.714 |
| 100-200 | 32 | 10 | | | 25 | 17 | 13 | 29 | <0.001 ^a |
| >200 | 17 | 3 | | | 15 | 5 | 1 | 19 | |
| TNM stage | | | | | | | | | |
| I | 14 | 14 | 6.294 | 0.043 ^a | 13 | 15 | 20 | 8 | 16.816 |
| II | 21 | 12 | | | 20 | 13 | 14 | 19 | <0.001 ^a |
| III | 28 | 7 | | | 22 | 13 | 7 | 28 | |
| Tumor position | | | | | | | | | |
| Upper | 12 | 16 | 9.167 | 0.010 ^a | 11 | 17 | 18 | 10 | 7.887 |
| Middle | 37 | 13 | | | 34 | 16 | 18 | 32 | 0.019 ^a |
| Lower | 14 | 4 | | | 10 | 8 | 5 | 13 | |

^aP<0.05. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2.

Table IV. Associations between EPHB4/Ephrinb2 and caspase-8 protein expression and the clinical and pathological features of individuals with esophageal cancer (n=96 pairs).

| Factors | n | EPHB4 | | | EFNB2 | | | Caspase-8 | | |
|----------------|----|-------------------------|--------|---------------------|------------|--------|--------------------|-----------|--------|---------------------|
| | | C/N | t/F | P-value | C/N | t/F | P-value | C/N | t/F | P-value |
| Sex | | | | | | | | | | |
| Male | 67 | 8.36±5.95 | -1.005 | 0.318 | 8.41±3.67 | 2.589 | 0.011 ^a | 0.27±0.54 | 1.592 | 0.115 |
| Female | 29 | 9.62±4.86 | | | 6.48±2.41 | | | 0.30±0.35 | | |
| Age, years | | | | | | | | | | |
| ≤35 | 2 | 10.33±0.94 | 2.044 | 0.135 | 10.33±0.94 | 2.044 | 0.135 | 0.28±0.54 | 0.832 | 0.438 |
| 35-50 | 22 | 6.65±3.43 | | | 6.65±3.43 | | | 0.27±0.40 | | |
| ≥50 | 72 | 9.34±6.11 | | | 9.34±6.11 | | | 0.28±0.44 | | |
| Metastasis | | | | | | | | | | |
| Yes | 57 | 10.06±6.05 ^a | -2.862 | 0.005 ^a | 8.66±3.38 | -2.987 | 0.004 ^a | 0.21±0.31 | 5.072 | <0.001 ^a |
| No | 39 | 6.82±4.41 | | | 6.61±3.20 | | | 0.38±0.47 | | |
| Family history | | | | | | | | | | |
| Yes | 55 | 10.26±6.03 | -3.181 | 0.002 ^a | 7.78±3.40 | -0.136 | 0.892 | 0.29±0.43 | 1.703 | 0.092 |
| No | 41 | 6.72±4.40 | | | 7.88±3.55 | | | 0.28±0.33 | | |
| Tumor size | | | | | | | | | | |
| I | 34 | 5.71±2.60 | 13.129 | <0.001 ^a | 6.50±2.59 | 4.174 | 0.018 ^a | 0.36±0.41 | 16.534 | <0.001 ^a |
| II | 42 | 9.23±4.33 | | | 8.47±3.23 | | | 0.25±0.31 | | |
| III | 20 | 12.89±8.49 | | | 8.73±4.50 | | | 0.21±0.23 | | |
| I vs. II | | | | 0.003 ^a | | | 0.012 ^a | | | <0.001 ^a |
| I vs. III | | | | <0.001 ^a | | | 0.020 ^a | | | <0.001 ^a |
| II vs. III | | | | 0.009 | | | 0.777 | | | 0.108 |
| Tumor stage | | | | | | | | | | |
| I | 28 | 5.91±2.50 | 5.882 | 0.004 ^a | 7.73±3.13 | 0.820 | 0.443 | 0.32±0.22 | 4.083 | 0.020 ^a |
| II | 33 | 9.33±4.56 | | | 7.32±3.33 | | | 0.28±0.26 | | |
| III | 35 | 10.46±5.65 | | | 8.38±3.79 | | | 0.25±0.49 | | |
| I vs. II | | | | 0.015 ^a | | | 0.642 | | | 0.308 |
| I vs. III | | | | 0.001 ^a | | | 0.460 | | | 0.006 ^a |
| II vs. III | | | | 0.385 | | | 0.208 | | | 0.070 |
| Tumor position | | | | | | | | | | |
| Upper | 28 | 6.65±3.12 | 2.986 | 0.055 | 7.44±3.23 | 0.278 | 0.758 | 0.28±0.43 | 1.337 | 0.268 |
| Middle | 50 | 9.83±6.66 | | 0.169 | 7.92±3.59 | | | 0.27±0.25 | | |
| Lower | 18 | 8.97±4.90 | | 0.570 | 8.17±3.49 | | | 0.31±0.31 | | |

Table IV. Continued.

| Factors | n | EPHB4 | | EFNB2 | | Caspase-8 | |
|------------------|---|-------|-----|---------|-----|-----------|---------|
| | | C/N | t/F | P-value | C/N | t/F | P-value |
| Upper vs. middle | | | | 0.560 | | | 0.114 |
| Upper vs. lower | | | | 0.490 | | | 0.272 |
| Middle vs. lower | | | | 0.797 | | | 0.877 |

Values expressed as mean \pm SD. t values are provided for comparisons of 2 groups, while F values are provided for comparisons between ≥ 3 groups. *P<0.05. EPHB4, EPH receptor B4; EFNB2, ephrin B2; C/N, the ratio of percentage of positively immunostaining cells in (C) esophagus cancer sample compared with (N) matched normal esophagus sample.

exhibited a significantly shortened median survival time (Table V). In addition, as presented in Table V, patients with a family history of cancer exhibited a significantly decreased survival time compared with those without a family history of cancer (P<0.001). Furthermore, older patients also had a shortened survival time compared with those patients that were younger (P<0.001), and patients with metastatic tumors exhibited a markedly decreased survival time compared with those without tumor metastasis (P<0.001) (Table V). However, there were no significant associations observed between survival and sex, tumor size, stage or position. The multivariate analysis results revealed that the mRNA expression level of *EPHB4* and *EFNB2*, the protein expression level of *EPHB4* and caspase-8, metastasis and family history were all significant independent risk factors for ESCC, with hazard ratios of 5.290, 3.146, 1.394, 2.784, 1.885 and 1.786, respectively (Table VI).

Discussion

A number of studies have reported that *EPHB4* and/or *EFNB2* expression is upregulated in multiple malignancies, including gastric (20), colon (21), uterine endometrial (22,23), breast (24), cervical (25) and ovarian cancer (26), melanoma (27), esophageal squamous cell carcinoma (14,16) and squamous cell carcinoma of the head and neck (28), which suggests that *EPHB4* and *EFNB2* may serve an oncogenic role in these tumor types. In the present study, it was observed that the expression of either *EPHB4* or *EPNB2* was increased in ESCC samples compared with that in corresponding normal esophageal tissues. It has been previously demonstrated that the upregulation of *EPHB4* or *EPNB2* is associated with metastasis and decreased survival in patients with ESCC (14,16); however, the present study revealed that it is also associated with tumor size and position, and family history, as well as confirming its association with decreased survival. Therefore, *EPHB4* and *EFNB2* may also serve oncogenic roles in the development and progression of ESCC.

The present study revealed that *EPHB4* expression exhibited a positive correlation with *EFNB2* expression, at both the mRNA and protein level; furthermore, IHC demonstrated that both molecules were expressed in the majority of ESCC cells. Considering they are cognate receptors and ligands, the aforementioned results suggested their potential ligation and the activation of downstream pathways in ESCC. Previous studies demonstrated that the activation of *EPHB4* and/or *EFNB2* triggered 'forward' and 'reverse' bidirectional signaling (2,3), which may stimulate angiogenesis *in vivo* (25,29-32), and stimulated the growth of primary and metastatic tumor cells (33,34). The *EPHB4* 'forward' signaling was able to promote the proliferation and migration of endothelial cells via the PI-3 kinase pathway, which increased the formation of new cancer vasculature (35). The *EFNB2* 'reverse' signaling, upon activation by *EPHB4*, not only induced an angiogenic response in cultured endothelial cells, but also promoted angiogenesis in breast cancer xenografts *in vivo* (35). In addition, *EPHB4* and *EFNB2* were also revealed to promote angiogenesis-independent tumor formation, in which the *EFNB2*-dependent *EPHB4* 'forward' signaling enhanced the migration and invasion of melanoma cells (36), via the activation of RhoA GTPase. The present study determined that

Table V. Univariate survival analysis of the association between expression levels of *EPHB4*, *EFNB2* and caspase-8 and certain clinicopathological characteristics in patients with esophageal cancer.

| Factors | Cases (n=96) | Events, n | Median survival, months | SE | Log-rank | P-value |
|-----------------------------|--------------|-----------|-------------------------|-------|----------|---------------------|
| Sex | | | | | 1.22 | 0.259 |
| Male | 67 | 47 | 35.585 | 1.179 | | |
| Female | 29 | 22 | 37.177 | 1.765 | | |
| Age, years | | | | | 47.37 | <0.001 ^a |
| <30 | 2 | 2 | 37.661 | 0.100 | | |
| 30-50 | 22 | 16 | 36.040 | 2.179 | | |
| >50 | 72 | 51 | 20.500 | 1.077 | | |
| Metastasis | | | | | 25.30 | <0.001 ^a |
| Yes | 57 | 36 | 28.868 | 1.624 | | |
| No | 39 | 33 | 40.579 | 1.079 | | |
| Family history | | | | | 15.95 | <0.001 ^a |
| Yes | 55 | 31 | 31.126 | 1.152 | | |
| No | 41 | 38 | 40.150 | 1.125 | | |
| Tumor size, cm ³ | | | | | 2.13 | 0.334 |
| ≤100 | 34 | 29 | 38.291 | 1.325 | | |
| 100-200 | 42 | 30 | 34.593 | 1.609 | | |
| >200 | 20 | 10 | 33.607 | 2.517 | | |
| TNM stage | | | | | 0.44 | 0.809 |
| I | 28 | 23 | 37.233 | 1.662 | | |
| II | 33 | 21 | 35.072 | 1.660 | | |
| III | 35 | 25 | 34.835 | 1.761 | | |
| Tumor position | | | | | 3.48 | 0.175 |
| Upper | 28 | 24 | 37.937 | 1.779 | | |
| Middle | 50 | 34 | 35.072 | 1.424 | | |
| Lower | 18 | 11 | 34.835 | 1.740 | | |
| <i>EPHB4</i> (mRNA) | | | | | 20.77 | <0.001 ^a |
| Low | 33 | 33 | 41.400 | 1.154 | | |
| High | 63 | 36 | 31.358 | 0.960 | | |
| <i>EFNB2</i> (mRNA) | | | | | 3.03 | 0.041 ^a |
| Low | 41 | 38 | 37.863 | 1.391 | | |
| High | 55 | 31 | 33.898 | 1.245 | | |
| Caspase-8 (mRNA) | | | | | 0.532 | 0.466 |
| Low | 37 | 33 | 48.500 | 3.911 | | |
| High | 59 | 36 | 52.815 | 3.307 | | |
| <i>EPHB4</i> (protein) | | | | | 7.420 | 0.006 ^a |
| Low | 48 | 36 | 39.525 | 1.274 | | |
| High | 48 | 33 | 30.168 | 1.342 | | |
| <i>EFNB2</i> (protein) | | | | | 2.715 | 0.095 |
| Low | 46 | 37 | 38.573 | 0.641 | | |
| High | 50 | 32 | 32.417 | 2.169 | | |
| Caspase-8 (protein) | | | | | 4.016 | 0.045 ^a |
| Low | 36 | 26 | 34.898 | 1.245 | | |
| High | 60 | 43 | 39.863 | 1.391 | | |

^aP<0.05. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2; TNM, Tumor-Node-Metastasis.

EPHB4 expression was associated with tumor size, metastasis and stage, indicating that *EPHB4* may influence ESCC cell

proliferation and migration. *EPHB4* has been reported to promote the proliferation and migration of tumor cells in a

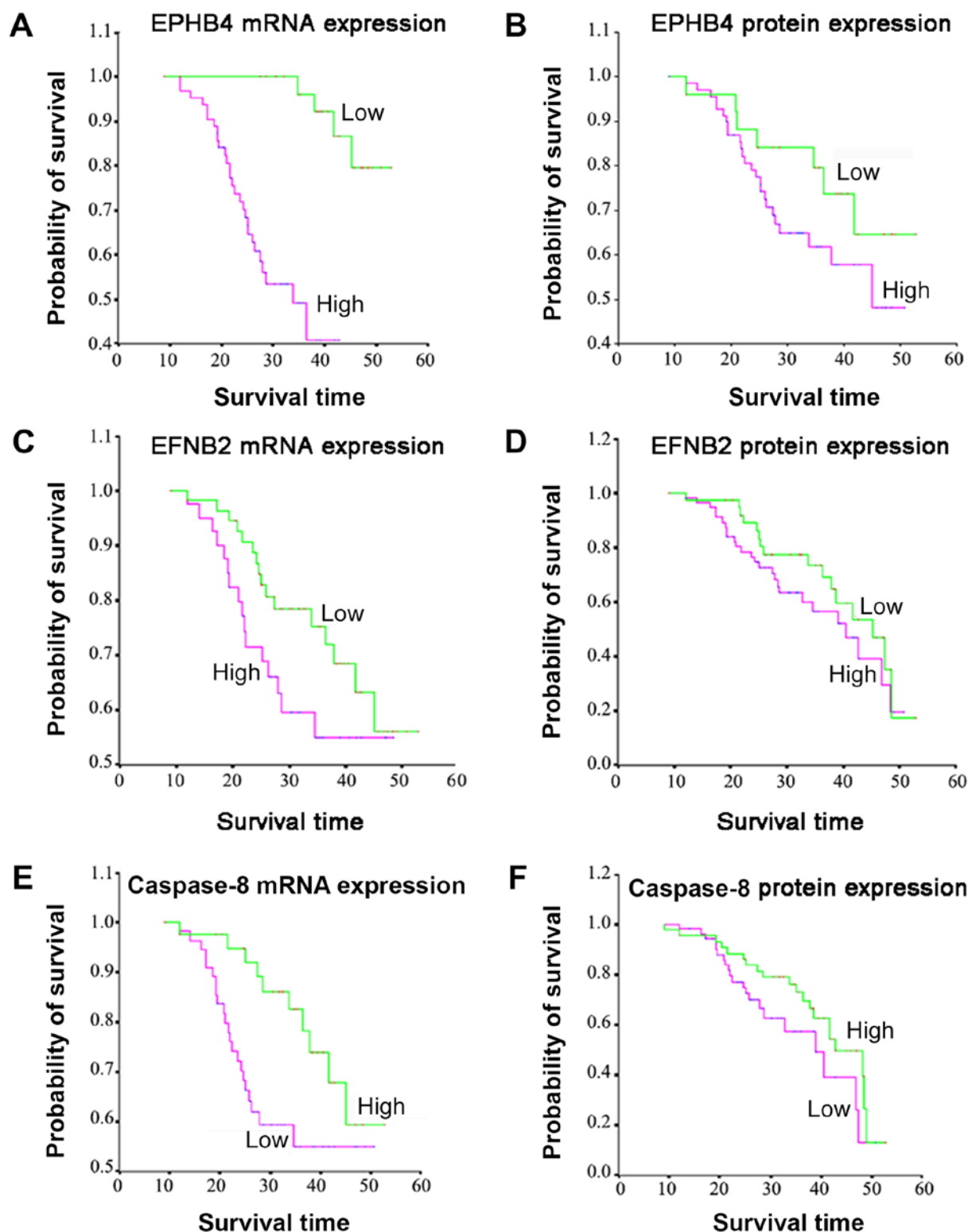


Figure 3. Survival curves starting from the time of diagnosis of patients with ESCC, and comparing OS times between patients with high and low expression levels of various proteins and mRNAs. Comparison of survival times between high and low *EPHB4* expression groups at the (A) mRNA and (B) protein levels. Comparison of survival times between high and low *EFNB2* expression groups at the (C) mRNA and (D) protein levels. Comparison of survival times between high and low caspase-8 expression groups at the (E) mRNA and (F) protein level. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2.

variety of different cancer types (22,23,28,36-42), which supports the results of the present study. In addition, the present study demonstrated that the upregulation of *EPHB4* and *EFNB2* was associated with poor outcome, and there have been similar reports in squamous cell carcinoma of the

head and neck (28), as well as in endometrial (22) and ovarian cancer (26,43).

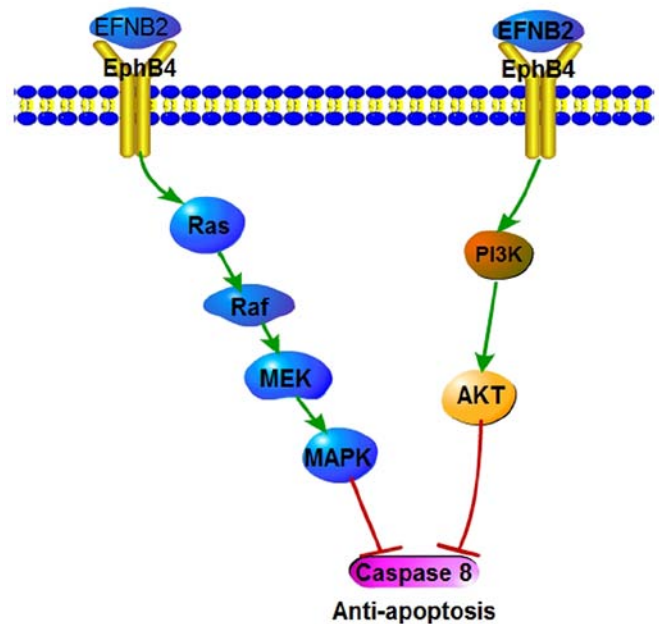
Resistance to apoptosis is required for tumor growth, and is a hallmark of cancer cells (44). Apoptosis resistance contributes to tumorigenesis, and results in the failure of cyto-

Table VI. Multivariate Cox proportional hazards regression analysis (n=96 pairs).

| Variables | Hazard ratio | 95% CI | P-value |
|-----------------------------|--------------|-------------|--------------------|
| Sex, male vs. female | 1.253 | 0.533-2.946 | 0.606 |
| Age, years | | | |
| 35-50 vs. ≤35 | 0.741 | 0.341-1.641 | 0.451 |
| ≥50 vs. ≤35 | 0.762 | 0.355-1.693 | 0.493 |
| Metastasis, yes vs. no | 1.885 | 1.545-2.517 | 0.037 ^a |
| Family history, yes vs. no | 1.786 | 1.217-2.389 | 0.026 ^a |
| Tumor size, cm ³ | | | |
| 100-200 vs. ≤100 | 1.472 | 0.668-2.115 | 0.107 |
| >200 vs. ≤100 | 1.662 | 0.715-2.262 | 0.227 |
| Tumor stage | | | |
| II vs. I | 1.001 | 0.441-1.379 | 0.110 |
| III vs. I | 1.009 | 0.449-1.382 | 0.172 |
| Tumor position | | | |
| Middle vs. upper | 0.915 | 0.473-1.771 | 0.752 |
| Lower vs. upper | 0.936 | 0.484-1.817 | 0.912 |
| High vs. low expression | | | |
| <i>EPHB4</i> (mRNA) | 5.290 | 3.723-7.706 | 0.012 ^a |
| <i>EFNB2</i> (mRNA) | 3.146 | 2.070-5.248 | 0.037 ^a |
| Caspase-8 (mRNA) | 0.936 | 0.323-2.713 | 0.903 |
| <i>EPHB4</i> (protein) | 1.394 | 1.011-1.968 | 0.035 ^a |
| <i>EFNB2</i> (protein) | 1.350 | 0.596-3.058 | 0.472 |
| Caspase-8 (protein) | 2.784 | 1.888-5.727 | 0.031 ^a |

^aP<0.05. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2; CI, confidence interval.

toxic therapies and a poor prognosis in patients, suggesting that targeting apoptotic pathways may represent a promising therapeutic approach for anticancer treatment. Accumulating evidence has demonstrated that apoptosis resistance, caused by downregulation of proapoptotic signaling molecules (such as caspase-8), frequently occurs in tumors of various origins. The present study demonstrated that the mRNA and protein level of caspase-8 was significantly downregulated in ESCC tissues compared with that in paracancerous tissues, indicating that this molecule may influence escape from endogenous growth control in the development and progression of ESCCs, which was similar to the findings previously reported (19). However, the present study also revealed that the expression of caspase-8 was associated with certain clinicopathological characteristics, including metastasis, tumor size, position and stage, and patient prognosis, in contrast to certain previously reported results (18). In conclusion, the downregulation of caspase-8 expression in ESCC suggested that it may serve as a useful predictor of prognosis in this type of cancer. Furthermore, the present study analyzed the associations between *EPHB4*, *EFNB2* and caspase-8 in ESCC. The results revealed that, in

Figure 4. Diagrammatic representation of the *EPHB4*/*EFNB2*-caspase 8 pathway. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2.

ESCC tissues, the expression levels of *EPHB4* and *EFNB2* were negatively correlated with caspase-8 at both the mRNA and protein levels, which, to the best of our knowledge, has not been yet reported elsewhere.

The present study indicates that the upregulation of *EPHB4* and *EFNB2* expression in tumor cells promotes growth (via the inhibition of apoptotic pathways), which may be facilitated by a decrease in caspase-8 expression, resulting from regulation of the downstream effectors of *EPHB4*/*EFNB2*. A diagram representing the underlying molecular mechanism concerning the role of *EPHB4*, *EFNB2* and caspase-8 in ESCC cells is exhibited in Fig. 4. The negative association between caspase-8 activation and *EPHB4* expression has been previously reported in ovarian carcinoma (26), and is consistent with the results of the present study. The Ras/MAPK/ERK and Akt signaling pathways, downstream of *EPHB4*, could confer anti-apoptotic characteristics. However, the molecular mechanism underlying the negative correlation between *EPHB4*/*EFNB2* and caspase-8 expression requires further investigation. Overall, the upregulation of *EPHB4* and *EFNB2* in tumor cells may disrupt caspase-8-mediated apoptosis and confer a survival advantage in tumor cells.

In summary, the present study reported that both *EPHB4* and *EFNB2* were upregulated, while caspase-8 was downregulated, in ESCC tissues compared with that in matched normal tissues. Expression levels were closely associated with a number of clinicopathological features, as well as patient survival. The current findings indicate the importance of the three molecules studied with regard to the genesis and progression of ESCC. Consequently, the expression levels of *EPHB4*, *EFNB2* and caspase-8 may serve as biological signatures and useful prognostic indicators in ESCC, as well as potentially representing novel therapeutic targets in this type of cancer.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Key R&D Program of China (grant no. 2018YFC1603002 and 2018YFC1604404); the 'Personalized Medicines-Molecular Signature-based Drug Discovery and Development', Strategic Priority Research Program of the Chinese Academy of Sciences (grant no. XDA12010316); the National Natural Science Foundation of China (grant nos. 31520103907, 81730083) to Dong Xie; the National Natural Science Foundation of China (grant nos. 31771538, 81972757); the Youth Innovation Promotion Association of the Chinese Academy of Sciences Fund and the Sanofi-SIBS 2018 Young Faculty Award; and Postdoctoral Science Foundation of China (grant no. 2017M622677).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XM, DX and JL conceived and designed the experiments. QN, BZ and PC performed the experiments. QN wrote the manuscript. QN collected and analyzed the data. PC assisted with revising the manuscript. All authors read and approved the final manuscript.

Ethical approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved by the Institutional Review Board of the Institute for Nutritional Sciences, Chinese Academy of Sciences (project number 30930023). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph Nomenclature Committee. *Cell* 90: 403-404, 1997.
- Wilkinson DG: Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev Neurosci* 2: 155-164, 2001.
- Palmer A and Klein R: Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. *Genes Dev* 17: 1429-1450, 2003.
- Heroult M, Schaffner F and Augustin HG: Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. *Exp Cell Res* 312: 642-650, 2006.
- Kd gsbrun M and Eichmann A: A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. *Cytokine Growth Factor Rev* 16: 535-548, 2005.
- Batlle E, Bacani J, Begthel H, Jonkheer S, Gregorieff A, van de Born M, Malats N, Sancho E, Boon E, Pawson T, *et al*: EphB receptor activity suppresses colorectal cancer progression. *Nature* 435: 1126-1130, 2005.
- Huuskio P, Ponciano-Jackson D, Wolf M, Kiefer JA, Azorsa DO, Tuzmen S, Weaver D, Robbins C, Moses T, Allinen M, *et al*: Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer. *Nat Genet* 36: 979-983, 2004.
- Okumura F, Joo-Okumura A, Obara K, Petersen A, Nishikimi A, Fukui Y, Nakatsukasa K and Kamura T: Ubiquitin ligase SPSB4 diminishes cell repulsive responses mediated by EphB2. *Mol Biol Cell* 8: 3532-3541, 2017.
- Teitz T, Lahti JM and Kidd VJ: Aggressive childhood neuroblastomas do not express caspase-8: An important component of programmed cell death. *J Mol Med (Berlin, Germany)* 79: 428-436, 2001.
- Teng Y, Dong YC, Liu Z, Zou Y, Xie H, Zhao Y, Su J, Cao F, Jin H and Ren H: DNA methylation-mediated caspase-8 down-regulation is associated with anti-apoptotic activity and human malignant glioma grade. *Int J Mol Med* 39: 725-733, 2017.
- Zhang Y: Epidemiology of esophageal cancer. *World J Gastroenterol* 19: 5598-5606, 2013.
- Siegel RL, Miller KD and Jemal A: Cancer Statistics, 2017. *CA Cancer J Clin* 67: 7-30, 2017.
- Domper Arnal MJ, Ferrandez Arenas A and Lanas Arbeloa A: Esophageal cancer: Risk factors, screening and endoscopic treatment in Western and Eastern countries. *World J Gastroenterol* 21: 7933-7943, 2015.
- Tachibana M, Tonomoto Y, Hyakudomi R, Hyakudomi M, Hattori S, Ueda S, Kinugasa S and Yoshimura H: Expression and prognostic significance of EFNB2 and EPHB4 genes in patients with esophageal squamous cell carcinoma. *Dig Liver Dis* 39: 725-732, 2007.
- Rice TW, Ishwaran H, Ferguson MK, Blackstone EH and Goldstraw P: Cancer of the esophagus and esophagogastric junction: An eighth edition staging primer. *J Thorac Oncol* 12: 36-42, 2017.
- Wang Y, Liu DP, Chen PP, Koeffler HP, Tong XJ and Xie D: Involvement of IFN regulatory factor (IRF)-1 and IRF-2 in the formation and progression of human esophageal cancers. *Cancer Res* 67: 2535-2543, 2007.
- Hasina R, Mollberg N, Kawada I, Mutreja K, Kanade G, Yala S, Surati M, Liu R, Li X, Zhou Y, *et al*: Critical role for the receptor tyrosine kinase EPHB4 in esophageal cancers. *Cancer Res* 73: 184-194, 2013.
- Takikita M, Hu N, Shou JZ, Wang QH, Giffen C, Taylor PR and Hewitt SM: Biomarkers of apoptosis and survival in esophageal squamous cell carcinoma. *BMC Cancer* 9: 310, 2009.
- Xue LY, Hu N, Song YM, Zou SM, Shou JZ, Qian LX, Ren LQ, Lin DM, Tong T, He ZG, *et al*: Tissue microarray analysis reveals a tight correlation between protein expression pattern and progression of esophageal squamous cell carcinoma. *BMC Cancer* 6: 296, 2006.
- Yin J, Cui Y, Li L, Ji J and Jiang WG: Overexpression of EPHB4 is associated with poor survival of patients with gastric cancer. *Anticancer Res* 37: 4489-4497, 2017.
- Liu W, Ahmad SA, Jung YD, Reinmuth N, Fan F, Bucana CD and Ellis LM: Coexpression of ephrin-Bs and their receptors in colon carcinoma. *Cancer* 94: 934-939, 2002.
- Alam S, Fujimoto J, Jahan I, Sato E and Tamaya T: Overexpression of ephrinB2 and EPHB4 in tumor advancement of uterine endometrial cancers. *Ann Oncol* 18: 485-490, 2007.
- Takai N, Miyazaki T, Fujisawa K, Nasu K and Miyakawa I: Expression of receptor tyrosine kinase EPHB4 and its ligand ephrin-B2 is associated with malignant potential in endometrial cancer. *Oncol Rep* 8: 567-573, 2001.
- Li X, Song C, Huang G, Sun S, Qiao J, Zhao J, Zhao Z and Li M: The coexpression of EPHB4 and EphrinB2 is associated with poor prognosis in HER2-positive breast cancer. *OncoTargets Ther* 10: 1735-1742, 2017.
- Zhang S, Jiang T and Liang M: Expression of Eph B4 and Ephrin B2 in cervical cancer tissues and angiogenesis. *Int J Gynaecol Obstet* 96: 46-47, 2007.

26. Kumar SR, Masood R, Spannuth WA, Singh J, Scehnet J, Kleiber G, Jennings N, Deavers M, Krasnoperov V, Dubeau L, *et al*: The receptor tyrosine kinase EphB4 is overexpressed in ovarian cancer, provides survival signals and predicts poor outcome. *Br J Cancer* 96: 1083-1091, 2007.
27. Neuber C, Belter B, Meister S, Hofheinz F, Bergmann R, Pietzsch HJ and Pietzsch J: Overexpression of receptor tyrosine kinase EPHB4 triggers tumor growth and hypoxia in A375 melanoma xenografts: Insights from multitracers small animal imaging experiments. *Molecules* 23: pii: E444, 2018.
28. Masood R, Kumar SR, Sinha UK, Crowe DL, Krasnoperov V, Reddy RK, Zozulya S, Singh J, Xia G, Broek D, *et al*: EPHB4 provides survival advantage to squamous cell carcinoma of the head and neck. *Int J Cancer* 119: 1236-1248, 2006.
29. Erber R, Eichelsbacher U, Powajbo V, Korn T, Djonov V, Lin J, Hammes HP, Grobholz R, Ullrich A and Vajkoczy P: EphB4 controls blood vascular morphogenesis during postnatal angiogenesis. *EMBO J* 25: 628-641, 2006.
30. Kertesz N, Krasnoperov V, Reddy R, Leshanski L, Kumar SR, Zozulya S and Gill PS: The soluble extracellular domain of EPHB4 (sEphB4) antagonizes EPHB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth. *Blood* 107: 2330-2338, 2006.
31. He S, Ding Y, Zhou J, Krasnoperov V, Zozulya S, Kumar SR, Ryan SJ, Gill PS and Hinton DR: Soluble EPHB4 regulates choroidal endothelial cell function and inhibits laser-induced choroidal neovascularization. *Invest Ophthalmol Vis Sci* 46: 4772-4779, 2005.
32. Noren NK, Lu M, Freeman AL, Koolpe M and Pasquale EB: Interplay between EPHB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. *Proc Natl Acad Sci USA* 101: 5583-5588, 2004.
33. Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1: 27-31, 1995.
34. Bouck N: Angiogenesis: A mechanism by which oncogenes and tumor suppressor genes regulate tumorigenesis. *Cancer Treat Res* 63: 359-371, 1992.
35. Steinle JJ, Meininger CJ, Forough R, Wu G, Wu MH and Granger HJ: Eph B4 receptor signaling mediates endothelial cell migration and proliferation via the phosphatidylinositol 3-kinase pathway. *J Biol Chem* 277: 43830-43835, 2002.
36. Yang NY, Pasquale EB, Owen LB and Ethell IM: The EPHB4 receptor-tyrosine kinase promotes the migration of melanoma cells through Rho-mediated actin cytoskeleton reorganization. *J Biol Chem* 281: 32574-32586, 2006.
37. Meyer S, Hafner C, Guba M, Flegel S, Geissler EK, Becker B, Koehl GE, Orso E, Landthaler M and Vogt T: Ephrin-B2 overexpression enhances integrin-mediated ECM-attachment and migration of B16 melanoma cells. *Int J Oncol* 27: 1197-1206, 2005.
38. Xia G, Kumar SR, Masood R, Koss M, Templeman C, Quinn D, Zhu S, Reddy R, Krasnoperov V and Gill PS: Up-regulation of EPHB4 in mesothelioma and its biological significance. *Clin Cancer Res* 11: 4305-4315, 2005.
39. Xia G, Kumar SR, Masood R, Zhu S, Reddy R, Krasnoperov V, Quinn DI, Henshall SM, Sutherland RL, Pinski JK, *et al*: EPHB4 expression and biological significance in prostate cancer. *Cancer Res* 65: 4623-4632, 2005.
40. Xia G, Kumar SR, Stein JP, Singh J, Krasnoperov V, Zhu S, Hassanieh L, Smith DL, Buscarini M, Broek D, *et al*: EPHB4 receptor tyrosine kinase is expressed in bladder cancer and provides signals for cell survival. *Oncogene* 25: 769-780, 2006.
41. Lian H, Jia X, Shi N, Xie S, Wang J, Wang W, Ma F, Liu H, Wang A, Cheng X and Liu C: Notch signaling promotes serrated neoplasia pathway in colorectal cancer through epigenetic modification of EPHB2 and EPHB4. *Cancer Manag Res* 10: 6129-6141, 2018.
42. Lv J, Xia Q, Wang J, Shen Q, Zhang J and Zhou X: EPHB4 promotes the proliferation, invasion, and angiogenesis of human colorectal cancer. *Exp Mol Pathol* 100: 402-408, 2016.
43. Wu Q, Suo Z, Kristensen GB, Baekelandt M and Nesland JM: The prognostic impact of EphB2/B4 expression on patients with advanced ovarian carcinoma. *Gynecol Oncol* 102: 15-21, 2006.
44. Hanahan D and Weinberg RA: The hallmarks of cancer. *Cell* 100: 57-70, 2000.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.