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

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Abstract. The upregulation of EPH receptor B4 (*EPHB4*) results in a survival advantage for tumor cells via the inhibition of the casapse-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.

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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

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 ≤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'-TCCTTCCTGCGGCTAAAC-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'- GATCATTGCTCCTCCTGAGC-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)=$level_{(target)}$/$level_{(\beta\text{-actin})}$=$2^{Cq(target)}/2^{Cq(\beta\text{-actin})}=2^{Cq(target)-Cq(\beta\text{-actin})}=2^{\Delta Cq}$. Furthermore, the relative levels (RL) of target genes in cancer tissues vs. corresponding normal samples were calculated according to the formula: RL=$NL_{(cancer)}/NL_{(normal)}=2^{\Delta Cq(cancer)}/2^{\Delta Cq(normal)}=2^{[\Delta Cq(cancer)-\Delta Cq(normal)]}=2^{\Delta\Delta Cq}$. As both NL and RL are represented as 2^{Cq}, the present study used ΔCq and ΔΔCq 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, x200). 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

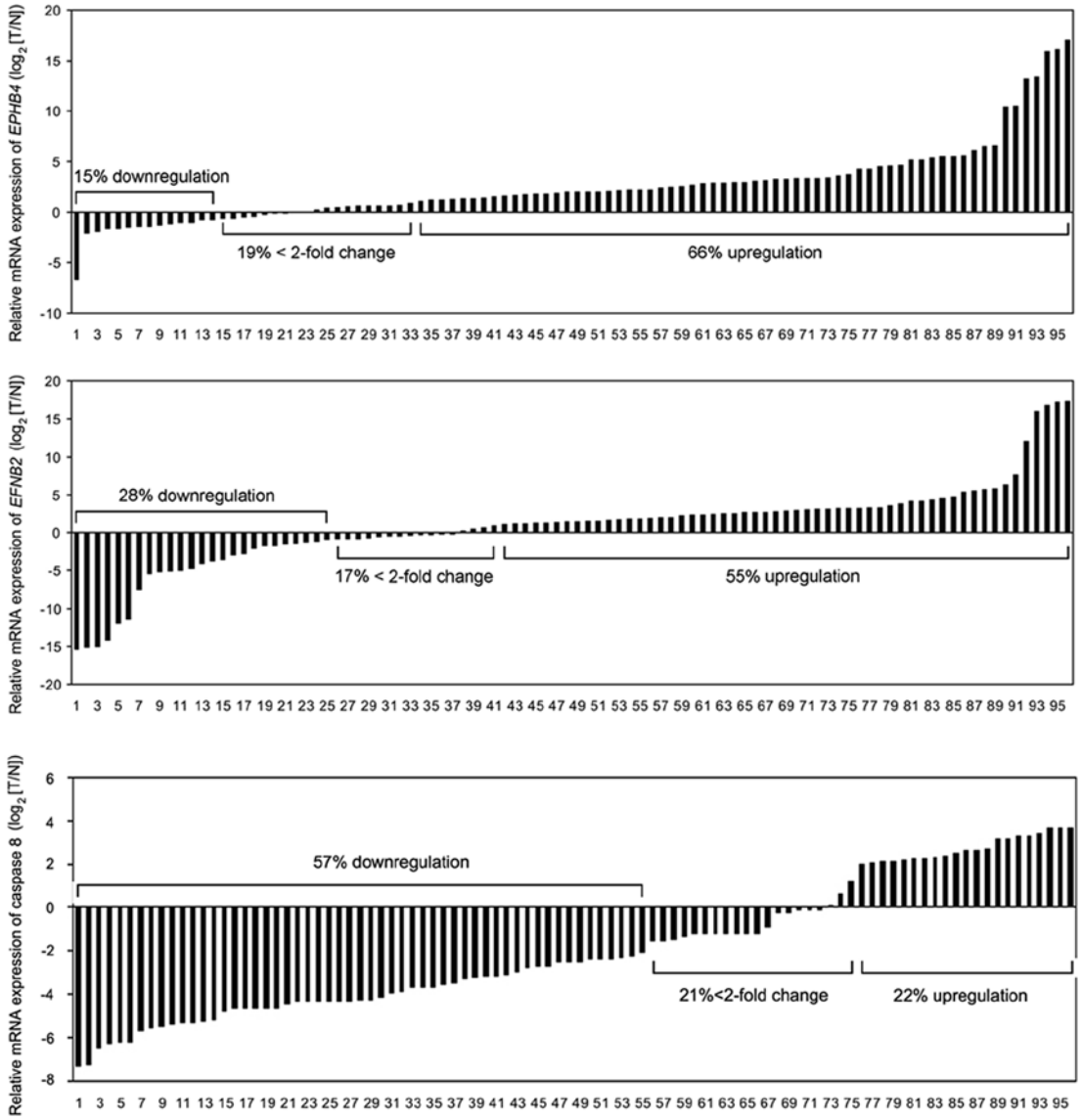

Figure 1. Expression patterns of EPHB4 and *EFNB2* in ESCC compared with those in matched normal esophageal tissues. Relative mRNA expression levels of *EPHB4* and *EFNB2* in human ESCC and paired paracancerous esophageal tissues were examined using quantitative PCR. Each bar is the $\log_2$ value of 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 $\text{Log}_2 2=1$, 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
EPHB4 mRNA	96	12.89±10.08	15.45±10.26	2.56±3.92	6.411	<0.01[a]
EFNB2 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]
EPHB4 protein	96	21.35±8.296	2.80±0.947	8.74±5.65	-21.603	<0.001[a]
EFNB2 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]

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

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).

	mRNA		Protein	
Genes	R-value	P-value	R-value	P-value
EPHB4 and *EFNB2*	0.620	<0.001[a]	0.202	0.049[a]
EPHB4 and caspase-8	-0.428	<0.001[a]	-0.340	0.001[a]
EFNB2 and caspase-8	-0.267	0.028[a]	-0.198	0.041[a]

[a]P<0.05. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2.

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

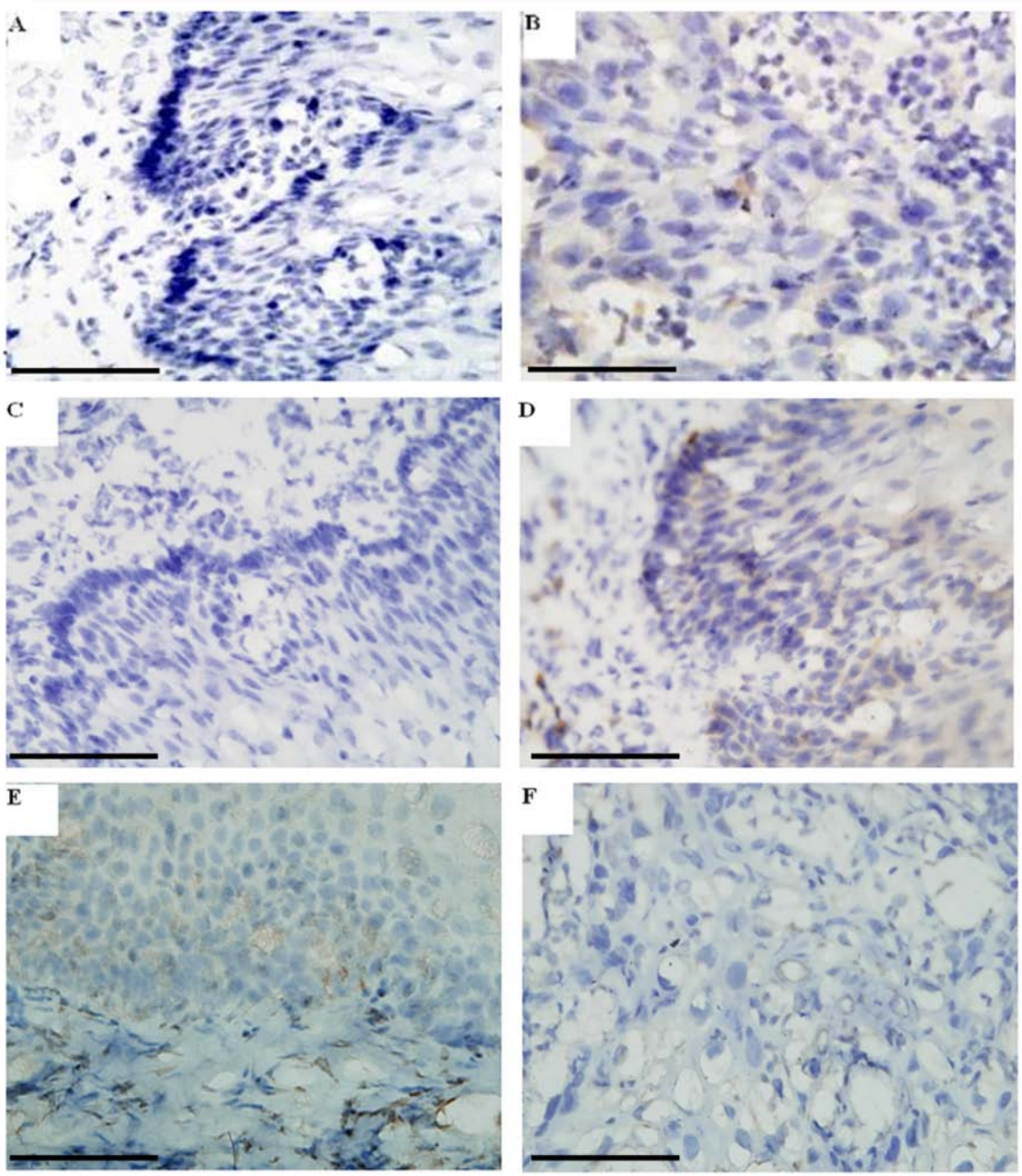

Figure 2. Representative immunohistochemical staining for *EPHB4 EFNB2* and caspase-8 proteins in ESCC and matched paracancerous esophageal tissues. (A) *EPHB4* and (C) *EFNB2* were observed in only a few cells in the normal esophageal tissues. (B) *EPHB4* and (D) *EFNB2* were expressed abundantly in 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 demonstrated 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).

	EPHB4				*EFNB2*				Caspase-8			
Factors	High, n	Low, n	χ^2	P-value	High, n	Low, n	χ^2	P-value	High, n	Low, n	χ^2	P-value
Sex			0.233	0.629			1.149	0.284			0.526	0.506
Male	45	22			36	31			26	41		
Female	18	11			19	10			15	14		
Age, years			2.433	0.303			2.001	0.368			1.733	0.494
≤35	2	0			2	0			1	1		
35-50	12	10			11	11			12	10		
≥50	49	23			42	30			28	44		
Metastasis			11.039	0.001[a]			0.970	0.325			41.552	<0.001[a]
Yes	45	12			20	19			9	48		
No	18	21			35	22			32	7		
Family history			53.940	<0.001[a]			15.668	<0.001[a]			7.327	0.012[a]
Yes	53	2			41	14			17	38		
No	10	31			14	27			24	17		
Tumor size, cm^3			14.415	0.001[a]			5.060	0.080			32.714	<0.001[a]
≤100	14	20			15	19			27	7		
100-200	32	10			25	17			13	29		
>200	17	3			15	5			1	19		
TNM stage			6.294	0.043[a]			1.942	0.379			16.816	<0.001[a]
I	14	14			13	15			20	8		
II	21	12			20	13			14	19		
III	28	7			22	13			7	28		
Tumor position			9.167	0.010[a]			6.075	0.048[a]			7.887	0.019[a]
Upper	12	16			11	17			18	10		
Middle	37	13			34	16			18	32		
Lower	14	4			10	8			5	13		

[a]P<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).

		EPHB4			EFNB2			Caspase-8		
Factors	n	C/N	t/F	P-value	C/N	t/F	P-value	C/N	t/F	P-value
Sex			-1.005	0.318		2.589	0.011[a]		1.592	0.115
Male	67	8.36±5.95			8.41±3.67			0.27±0.54		
Female	29	9.62±4.86			6.48±2.41			0.30±0.35		
Age, years			2.044	0.135		2.044	0.135		0.832	0.438
≤35	2	10.33±0.94			10.33±0.94			0.28±0.54		
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			-2.862	0.005[a]		-2.987	0.004[a]		5.072	<0.001[a]
Yes	57	10.06±6.05[a]			8.66±3.38			0.21±0.31		
No	39	6.82±4.41			6.61±3.20			0.38±0.47		
Family history			-3.181	0.002[a]		-0.136	0.892		1.703	0.092
Yes	55	10.26±6.03			7.78±3.40			0.29±0.43		
No	41	6.72±4.40			7.88±3.55			0.28±0.33		
Tumor size			13.129	<0.001[a]		4.174	0.018[a]		16.534	<0.001[a]
I	34	5.71±2.60			6.50±2.59			0.36±0.41		
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			5.882	0.004[a]		0.820	0.443		4.083	0.020[a]
I	28	5.91±2.50			7.73±3.13			0.32±0.22		
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			2.986	0.055		0.278	0.758		1.337	0.268
Upper	28	6.65±3.12		0.017[a]	7.44±3.23			0.28±0.43		
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.

		EPHB4			EFNB2			Caspase-8		
Factors	n	C/N	t/F	P-value	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 ± SD. t values are provided for comparisons of 2 groups, while F values are provided for comparisons between ≥3 groups. [a]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 Pl-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		
EPHB4 (mRNA)					20.77	<0.001[a]
Low	33	33	41.400	1.154		
High	63	36	31.358	0.960		
EFNB2 (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		
EPHB4 (protein)					7.420	0.006[a]
Low	48	36	39.525	1.274		
High	48	33	30.168	1.342		
EFNB2 (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		

[a]P<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

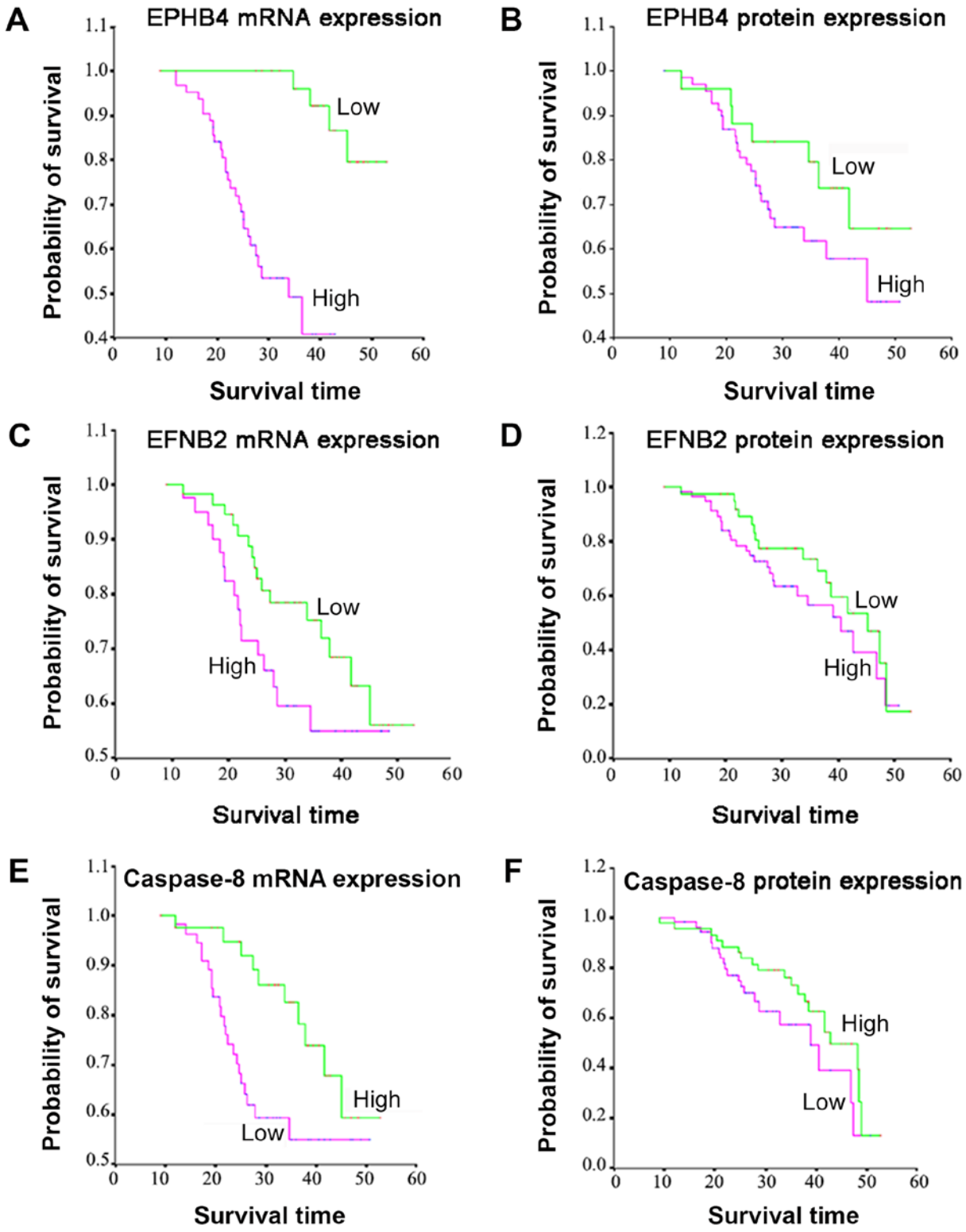

Figure 3. Survival curves starting from the time of diagnosis of patients with ESCC, and comparing OS times between patients with high and low expression levels of various proteins and mRNAs. Comparison of survival times between high and low *EPHB4* expression groups at the (A) mRNA and (B) protein levels. 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			
EPHB4 (mRNA)	5.290	3.723-7.706	0.012[a]
EFNB2 (mRNA)	3.146	2.070-5.248	0.037[a]
Caspase-8 (mRNA)	0.936	0.323-2.713	0.903
EPHB4 (protein)	1.394	1.011-1.968	0.035[a]
EFNB2 (protein)	1.350	0.596-3.058	0.472
Caspase-8 (protein)	2.784	1.888-5.727	0.031[a]

[a]P<0.05. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2; CI, confidence interval.

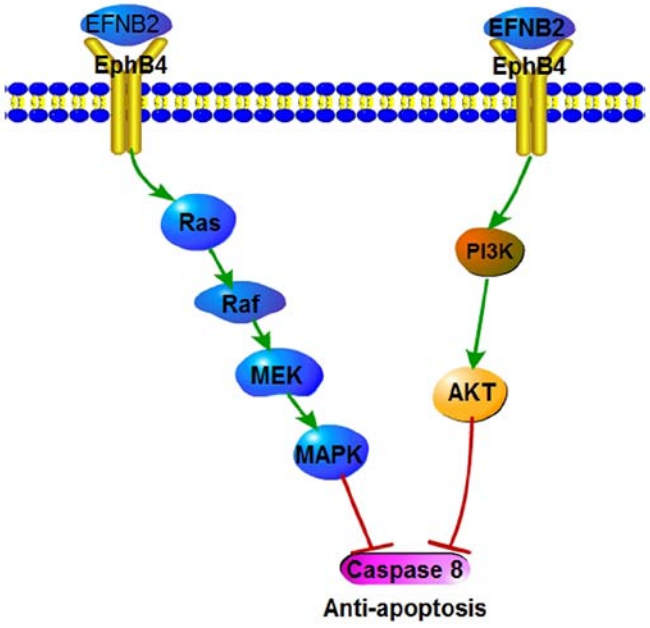

Figure 4. Diagrammatic representation of the *EPHB4*/*EFNB2*-caspase 8 pathway. *EPHB4*, EPH receptor B4; *EFNB2*, ephrin B2.

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 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.

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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.

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