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Predictive Value of EphA2 and EphrinA-1 Expression in Oesophageal Squamous Cell Carcinoma

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Abstract. *The aim of this study was to analyse the protein and mRNA expressions of EphA2 and EphrinA-1 in oesophageal squamous cell carcinomas and to explore their clinicopathological associations and predictive values in oesophageal squamous cell carcinoma. Tissue array and immunohistochemistry were used to assess the protein expressions of EphA2 and EphrinA-1 in tumours from 173 patients with oesophageal squamous cell carcinoma. Paraffin sections from 20 cases in which the tumours showed variable EphA2 and EphrinA-1protein expressions were used for laser capture microdissection and processed for RT-PCR detection of EphA2 and EphrinA-1 mRNA. Among the173 oesophageal squamous cell carcinomas, 33 (19.1%) were negative, 44 (25.4%) weakly-positive, 58 (33.5%) moderately-positive and 38 (22.0%) strongly-positive for EphA2 immunostaining. For EphrinA-1 protein expression, 27 tumours (15.6 %) were negative, 41 (23.7 %) weakly-positive, 80 (46.2 %) moderately-positive and 25 (14.5%) strongly-positive. EphA2 and EphrinA-1 were often co-localized in the same tumour areas and vascular endothelial cells. Variable amounts of EphA2 and EphrinA-1 mRNAs were observed in the 20 tumours analysed. No significant association was observed between EphA2 and EphrinA-1 protein expressions and age, tumour location, tumour size, histological differentiation or clinical stage. However, there was a significant correlation between EphA2 expression and lymph node metastases ($p<0.001$). In univariate analysis, high levels of EphA2/EphrinA-1 protein expression, higher number of lymph node metastasis, higher histological grade and clinical stage were significantly associated with shorter overall survival. In Cox multivariate analysis, only EphA2, number of lymph node metastasis and clinical stage were of independent significance. We conclude that EphA2 protein expression is confirmed to be of predictive value for unfavourable survival for oesophageal cancer patients and may be a good target for oesophageal cancer therapy.*

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Oesophageal cancer is one of the most frequently developed cancers in the world (1) and is a common cancer in the Chinese population (2, 3). The annual incidence rate in the world varies from $3/10^5$ to $64/10^5$, depending upon genetic vulnerability, diet and environmental factors. Although surgery is a curative treatment for early stage oesophageal cancer, it is still a limited clinical option since most patients present with advanced disease (4). The survival rate at 5 years was reported to be about 10%, although recent advancements in both surgical and adjuvant therapies have improved the 5-year survival rate to about 40% (5). Therefore, determination of prognostic factors in oesophageal cancer is important to commence proper treatment and to increase survival.

Receptor tyrosine kinases (RTK) have been proven to play an important role in cancer development and progression. It is clear that protein tyrosine phosphorylation of RTK generates powerful signals necessary for growth, migration and invasion of malignant cells (6). A number of RTKs are correlated with tumour progression and development (7). Among the RTKs, the largest family is EPH (erythropoietin-producing hepatoma cell line) (8). The most studied EPH family member is EphA2. The cellular functions of EphA2 in normal epithelia are not well understood, but work with

Table I. *Results of EphrinA-1 and EphA2 expressions in relation to clinicopathological characteristics in ESCC.*

Parameters	Total	EphA2 expression (%)					EphrinA-1 expression (%)				
	no.	Staining scores				*p*	Staining scores				*p*
		0	1	2	3		0	1	2	3	
Age											
≤50	52	12 (23.1)	14 (26.9)	17 (32.7)	9 (17.3)	0.38	8 (15.4)	11 (21.1)	25 (48.1)	8 (15.4)	0.86
51-60	50	6 (12.0)	14 (28.0)	14 (28.0)	16 (32.0)		9 (18.0)	9 (18.0)	24 (48.0)	8 (16.0)	
>60	71	15 (21.1)	16 (22.6)	27 (38.0)	13 (18.3)		10 (14.1)	21 (29.6)	31 (43.6)	9 (12.7)	
Gender											
Male	105	15 (14.3)	34 (32.4)	33 (31.4)	23 (21.9)	0.035	13 (12.4)	31 (29.5)	46 (43.8)	15 (14.3)	0.114
Female	68	18 (26.5)	10 (14.7)	25 (36.8)	15 (22.0)		14 (20.6)	10 (14.7)	34 (50.0)	10 (14.7)	
Differentiation											
Well	48	12 (25.0)	12 (25.0)	17 (35.4)	7 (14.6)	0.362	8 (16.7)	9 (18.7)	24 (50.0)	7 (14.6)	0.694
Moderate	74	11 (14.9)	18 (24.3)	30 (40.5)	15 (20.3)		8 (10.8)	21 (28.4)	34 (45.9)	11 (14.9)	
Poor	51	10 (19.6)	14 (27.5)	12 (23.5)	15 (29.4)		11 (21.6)	11 (21.6)	22 (43.1)	7 (13.7)	
Location											
Upper	18	2 (11.1)	6 (33.3)	4 (22.3)	6 (33.3)	0.476	2 (11.1)	3 (16.7)	12 (66.7)	1 (5.5)	0.285
Mid-thora	119	23 (19.3)	26 (21.8)	46 (38.7)	24 (20.2)		18 (15.1)	27 (22.7)	58 (48.7)	16 (13.5)	
Lower	36	8 (22.2)	11 (30.6)	9 (25.0)	8 (22.2)		7 (19.4)	10 (27.8)	11 (30.6)	8 (22.2)	
Size											
≤33	68	13 (19.1)	18 (26.5)	24 (35.3)	13 (19.1)	0.987	9 (13.2)	17 (25.0)	32 (47.1)	10 (14.7)	0.897
34-66	90	18 (20.0)	22 (24.5)	29 (32.2)	21 (23.3)		15 (16.7)	19 (21.1)	42 (46.6)	14 (15.6)	
>66	15	2 (13.3)	4 (26.7)	5 (33.3)	4 (26.7)		3 (20.0)	5 (33.3)	6 (40.0)	1 (6.7)	
Number of lymph node metastases											
0	95	29 (30.5)	29 (30.5)	24 (25.3)	13 (13.7)	0.0001	20 (21.1)	25 (26.3)	40 (42.1)	10 (10.5)	0.087
1	49	3 (6.1)	6 (12.2)	26 (53.1)	14 (28.6)		4 (8.2)	9 (18.4)	25 (51.0)	11 (22.4)	
2	28	1 (3.6)	9 (32.1)	8 (28.6)	10 (35.7)		3 (10.7)	7 (25.0)	15 (53.6)	3 (10.7)	
3	1	0	0	0	1 (100)		0	0	0	1 (100)	
UICC stage											
I	10	3 (30.0)	(50.0)	1 (10.0)	1 (10.0.)	0.45	3 (30.0)	2 (20.0)	4 (40.0)	1 (10.0)	0.143
II	77	12 (15.6)	17 (22.1)	31 (40.2)	17 (22.1)		7 (9.1)	18 (23.4)	37 (48.1)	15 (19.4)	
III	81	17 (21.0)	21 (25.9)	24 (29.6)	19 (23.5)		17 (21.0)	17 (21.0)	38 (46.9)	9 (11.1)	
IV	5	1 (20.0)	1 (20.0)	2 (40.0)	1 (20.0)		1 (20.0)	4 (80.0)	0	0	

tumour-based models suggests its potential role in regulation of cell growth, survival, migration and angiogenesis (9-13). EphA2 expression is frequently elevated in cancer. High levels of EphA2 in tumour cells have been reported using multiple and diverse cell models and clinical specimens, including breast cancer (14), colon cancer (10), prostate cancer (15), non-small cell lung cancers (16) and aggressive melanomas (17). However, EphA2 does not appear to simply function as a marker but as an active participant in malignant progression. To date, associations between tumour EphA2 expression and clinicopathological features and clinical outcome have not been fully characterised, especially for oesophageal squamous cell carcinoma (ESCC), where only a few studies were reported (18).

EphrinA-1 was originally found in human umbilical vein endothelial cells (HUVEC) in a tumour necrosis factor α (TNFα) model (19). The EphA2 receptor plays a critical role in TNFα-induced neovascularization because TNFα up-regulates Ephrin-A1, which causes receptor activation in blood vessels (20). EphrinA-1 also interacts with all the other EphA subclass receptors but with different affinities (21). It has been found that EphA2 and Ephrin-A1 are co-expressed in several types of human malignant tumour cells as well as in blood vessels (20), indicating that Ephrin-A1 and EphA2 jointly play a role in tumour development, at least in part by influencing tumour neovascularization.

In this study, we systematically analysed the protein expressions of EphA2 and EphrinA-1 in a series of 173 ESCC by immunohistochemistry and mRNA expression by Laser Capture Microdissection (LCM)-assisted RT-PCR. The results were correlated with classic clinicopathogical factors and overall survival.

Table II. *Primers used for RT-PCR.*

Primers	Genebank access no.	Primer location and sequences (bp)	Exon region	Product size (bp)	Annealing temperature (˚C)
EphA2	NM_004431	F: 898 ~ 920 5'GCCAGGCAGGCTACGAGAAGGTG 3'(23)	3 ~ 6	586	60
		R: 1483 ~ 1460 5'CTCCAGGAGACGCTAAGCGAGGTG 3'(24)			
EphrinA-1	NM_004428	F: 597 ~ 616 5' GAGACTTGCAGCAGATGACC 3'(20)	4 ~ 5	486	60
		R: 1082 ~ 1065 5'GACAAGCTTTGCCCATCC 3'(18)			
GAPD	NM_002046	F: 466 ~ 483 5'TTCGTCATGGGTGTGAAC 3'(18)	5 ~ 7	297	60
		R: 762 ~ 744 5'AGTGAGCTTCCCGTTCAGC 3'(19)			

Materials and Methods

Patients and specimens. In total, 173 patients with ESCC were included in this study. The patients were treated at Anyang Tumour Hospital, Henan Province, China, during the period 1991-1994. Of the 173 patients, 105 were men and 65 were women, aging from 33 -73 years (mean age: 53.6 years). All patients underwent potentially curative surgery without preoperative therapy. The tumour stage was classified according to the 5th edition of the TNM Classification of the International Union against Cancer (22). Patient and tumour parameters are listed in Table I. All of the patients were followed at the Anyang Tumour Hospital until May 2004. A total of 93 (53.8%) patients died during the follow-up.

Surgically removed specimens were routinely fixed in buffered formalin and embedded in paraffin block for clinical diagnosis and reclassified for this study.

Immunohistochemistry. Immunohistochemical staining was performed with the Optimax® Automated Cell Staining System Plus (BioGenex, San Ramon, CA, USA). Each 4-μm section cut from both archived paraffin and tissue array blocks was immunohistochemically analysed simultaneously, using the polyclonal rabbit antibodies EphA2 (sc-924, 1:400, 0.5 μg IgG/ml) and EphrinA-1 (sc-911, 1:300, 0.7 μg IgG/ml) (both from Santa Cruz Biotechnology, Inc., CA, USA) for 30 minutes at room temperature. The sections were then incubated with peroxidase-labelled polymer conjugated to goat anti-rabbit IgG for 30 minutes before staining for 5 minutes with 3'3-diaminobenzidine tetrahydrochloride (DAB). They were then counterstained with hematoxylin, dehydrated and mounted in Diatex. Sections of the human breast carcinomas known to express EphA2 and EphrinA-1 were included in each staining series as positive controls, while negative controls included substitution of the polyclonal antibody with normal rabbit IgG of the same concentration as the polyclonal antibody. All controls gave satisfactory results.

Evaluation of staining for EphA2 and EphrinA-1. The immunoreactivity was evaluated according to the intensity of staining and scored using a 0-3 scale (0: no staining or negative; 1: weak staining; 2: moderate staining; 3: strong staining). All slides were read independently by three experienced observers who were unaware of the related clinical information. Differences in immunohistochemical scores were rare and resolved by consensus.

Tissue array. A multitissue array block was made with the MTA-1 manual tissue arrayer (Beecher Instruments Inc., Sun Prairie, WI, USA). Five-micron sections from the routinely made paraffin blocks were stained with H&E and were re-evaluated to confirm the diagnosis and to identify representative areas of the specimen. The related paraffin blocks were subsequently oriented and marked. From these blocks, tissue cores with a diameter of 0.6 mm were punched and arrayed in triplicate on a recipient paraffin block.

After block construction was completed, the block was placed into a 40˚C oven overnight to tighten the cylinders by slightly melting the paraffin. Five-micron sections of these tissue array blocks were cut and placed on charged Super-Frost Plus glass slides, and dried in a 60˚C oven for 2-4 hours. These sections were used for immunohistochemical staining, as described above.

Laser capture microdissection (LCM). The PixCell LCM system (Arcturus Bioscience, Inc., Mountain View, CA, USA) was used to microdissect ESCC from H&E-stained and Paraffin-embed tissues from 20 tumours immunohistochemically proven to have different levels of EphA2/EphrinA-1 protein expressions. For each specimen, about 200 cells were microdissected and collected. Briefly, ten-micron-thick sections were cut using disposable microtome blades, placed on uncharged glass slides and stored at room temperature for 2-4 hours, followed by deparaffinisation with xylene for 2 x 3 minutes, 100% ethanol for 2 x 2 minutes, 2,95% ETOH for 2 x 2 minutes, then rinsed in distilled water, followed by H&E staining. The slides were dehydrated with 95% ethanol and 100% ethanol, incubated in xylene for 2 x 3 minutes, air dried, and used for LCM. Small tumour areas (about 200 cells) on the section were selected and laser captured. The LCM parameters were as follows: laser power of 70 milliwatts, laser pulse duration of 1.2-3.5 milliseconds and laser spot size of 7.5-15 μm diameters. Complete capture was defined as capture of more than 90% of the tissue within the laser-activated capture area without transfer of any tissue outside the capture area. The tissue section was overlaid with a thermoplastic polymer membrane mounted on an optically transparent cap (Arcturus Bioscience, Inc.).

RNA extraction. About 200 cells captured on the cap were microcentrifuged into an Eppendorf tube. Total RNA was then extracted by using an Absolutely RNA™ Nanoprep Kit (Stratagene Inc., La Jolla, CA, USA), according to the protocol recommended by the manufacturer.

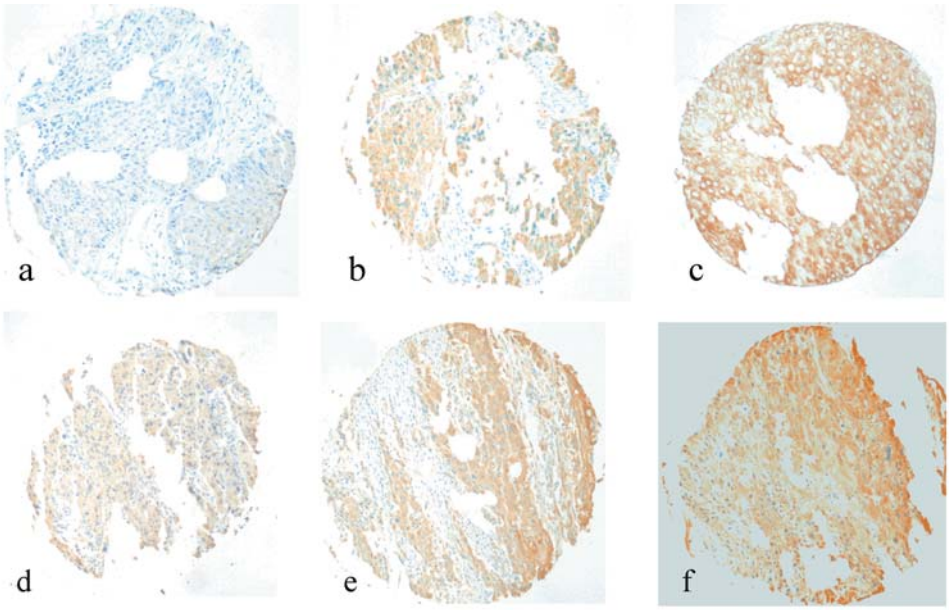

Figure 1. *Tissue array immunohistochemistry showing negative (a), mild (b) and strong (c) EphrinA-1 expression and weak (d), mild (e) and strong (f) EphA2 expression in ESCC.*

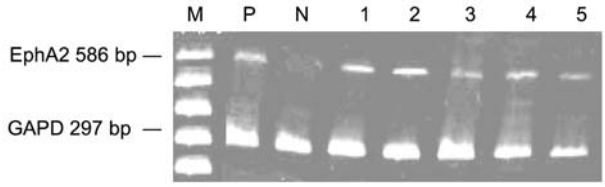

Figure 2. *RT-PCR result of EphA2 mRNA in ESCC. (M): 100 bp ladder marker; (P): positive control; (N): negative control; Lanes 1-5 showing different EphA2 mRNA expressions.*

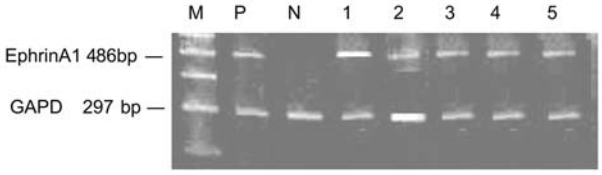

Figure 3. *RT-PCR result of EphrinA-1 mRNA in ESCC. (M):100 bp ladder marker; (P): positive control; (N): negative control; Lanes 1-5 showing variable EphrinA-1 mRNA expressions.*

RT-PCR. Qiagen OneStep (Qiagen Inc., Valencia, CA, USA) was used for RT-PCR, which was performed in a 25 μl volume using 5 μl 5 x Qiagen OneStep RT-PCR buffer, 5 μl 5 x Q solution, 200 μM dNTP, 0.6 μM 3' primer, 0.6 μM 5' primer, 0.2 μM 3' GAPD primer, 0.2 μM 5' GAPD primer, 2 μl RNA and 1 μl Qiagen OneStep RT-PCR enzyme mix. The reactions were carried out under the following conditions: 50˚C for 30 minutes for reverse transcription of RNA followed by 95˚C for 15 minutes for cDNA denaturation, and then 40 cycles at 94˚C for 60 seconds, 60˚C for 30 seconds and 72˚C for 1 minute. A final extension was performed at 72˚C for 7 minutes. The features of all primers are given in Table II. All primers span at least one intron. GAPD primers were used in each reaction as internal controls. Breast cancer cell line MDA-MB-231 cells were used as positive control and negative controls were performed using water instead of primer.

Statistical analysis. The association between protein expression and clinicopathological parameters was tested by the χ^2 test. Overall survival was calculated from date of surgery to date of death or May 1, 2004. Survival curves were then plotted with the Kaplan-Meier method. Survivals of different groups were compared by the log-rank test. The SPSS version 10.0 statistical package (SPSS Inc., Chicago,IL, USA) was used for the statistical analysis. *P*-values of <0.05 were considered statistically significant.

Results

EphA2 immunoreactivity. Positive EphA2 immunostaining was observed in the cytoplasm of cancer cells, lymphocytes and endothelial cells. The EphA2 immunoreactivty was quite homogeneous (Figure 1a). Expression in cancer cells was seen in 140 tumours (80.9%), among which 44 (25.4%) were weakly-positive, 58 (33.5%) moderately-positive and 38 (22.0%) strongly-positive (Table I). EphA2 immunoreactivity was found in the majority of the vascular endothelial cells and the intensity was consistent with or stronger than that in the cancer cells.

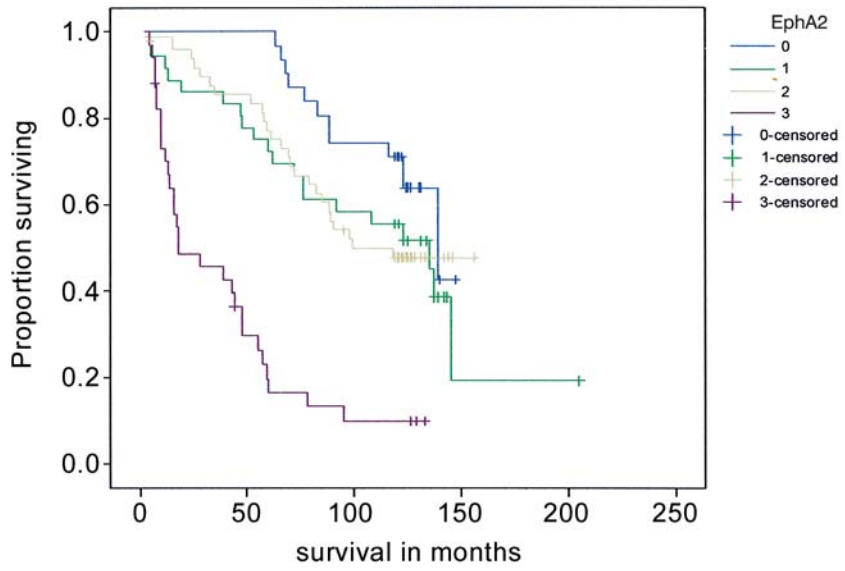

Figure 4. *Kaplan Meier plot of the effect of EphA2 protein expression on overall survival for patients with ESCC ($p<0.001$). Patients still alive or dead from causes other than ESCC are marked with a cross on the curves. Patients with tumours showing high levels of EphA2 protein expression show shorter overall survival than those with EphA2-negative expression.*

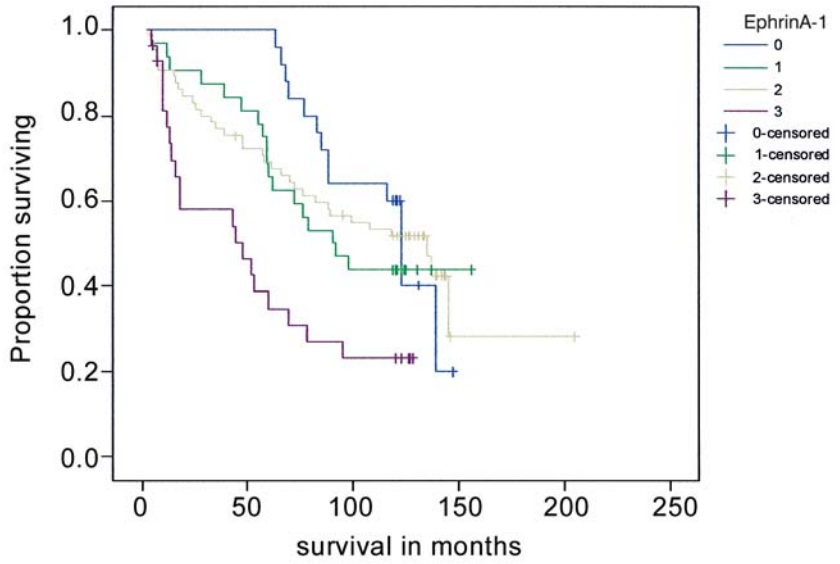

Figure 5. *Kaplan Meier plot of the effect of EphrinA-1 protein expression on overall survival for patients with ESCC ($p=0.017$). Patients still alive or dead from causes other than ESCC are marked with a cross on the curves. Patients with tumours with high levels of EphrinA-1 protein expression show shorter overall survival than those with EphrinA-1-negative expression.*

EphrinA-1 immunoreactivity. Immunostaining for EphrinA-1 was observed in the cytoplasm of cancer cells, lymphocyte and endothelial cells. Staining results for EphrinA-1 are provided in Table I. The immunostaining pattern of EphrinA-1 was also homogeneous (Figure 1 b) and the immunostaining was seen in nearly all endothelial cells of the blood vessels, but the intensity was weaker than that in cancer cells.

Correlation between EphA2 and EphrinA-1 protein expressions. EphA2 and EphrinA-1 protein expressions were co-localized in the same tumour areas and vascular endothelial cells with similar staining intensity (Figure 1 c, d) and their expressions were significantly associated ($p<0.001$).

Relationship to clinicopathological features. EphA2 and EphrinA-1 protein expressions were not associated with

Table III. *Multivariate analysis of factors of independent prognostic significance in ESCC.*

	p-value	Relative hazard	95% confidence limits
EphA2 protein expression	0.001	1.800	1.279-2.534
No. of lymph node metastasis	0.037	1.387	1.019-1.888
Histological grade	0.087	1.283	0.965-1.707
EphrinA-1 protein expression	0.770	0.953	0.690-1.316
Clinical stage	0.011	1.528	1.102-2.118

age, tumour location, tumour size, histological grade or cinical stage. The relationship between patients' clinicopathological factors and protein expression is summarised in Table I. Negative EphA2 protein expression was seen more often in tumour in women ($p=0.035$), and higher levels of EphA2 protein expression were significantly correlated with higher number of lymph node metastases ($p<0.001$).

EphA2 and EphrinA-1 mRNA expressions. The 20 selected samples were successfully examined using LCM-assisted RT-PCR. The intensity of the amplified band of EphA2 and EphrinA-1 in each sample was analysed and compared with that of the GAPD (glyceraldehyde-3-phosphate dehydrogenase) PCR band, which was added into the same RT-PCR reaction system. Of the 20 tumours, 8 were immunohistochemically scored as grade 3 for EphA2, 5 as grade 2, 3 as grade 1 and 4 were negative. Tumours with positive immunostaining for EphA2 had higher mRNA levels than those with negative staining, but no difference was observed in relation to the intensity of staining within the positive cases (Figure 2), indicating that mRNA expression did not fully correspond to its protein expression in ESCC.

For EphinA-1 protein expression, 8 of the 20 tumours were immunohistochemically scored as grade 3 for EphrinA-1, 6 as grade 2, 2 as grade 1 and 4 were negative. Tumours with positive immunostaining for EphrinA-1 had higher mRNA levels than those with negative immunostaining. Similarly, however, no difference was seen in relation to the intensity of staining in the positive cases (Figure 3).

Relation to survival. In univariate analysis, a significantly shorter overall survival was associated with higher histological grade ($p=0.017$), higher clinical stage ($p=0.003$), higher levels of EphA2 protein expression ($p<0.001$, Figure 4), higher levels of EphrinA-1 protein expression ($p=0.017$, Figure 5) and higher number of lymph node metastasis ($p<0.001$).

In Cox multivariate analysis with clinical stage, histological grade, number of lymph node metastasis, EphA2 and EphrinA-1 expressions included, clinical stage, number of lymph node metastasis and EphA2 were of independent significance for overall survival, as shown in Table III. Histological grade and EphrinA-1 were no longer significantly associated with overall survival in this Cox multivariate analysis (Table III).

Discussion

The immunohistocehmical results in our study suggest that EphA2 protein expression is correlated with gender. Negative EphA2 protein expressions in tumours from males and females were 14.3% and 26.5% of the ESCCs, respectively, and the difference was statistically significant ($p=0.035$). EphA2 protein expression was also significantly associated with the number of lymph node metastases ($p<0.001$). In addition to histological grade, number of lymph node metastasis and clinical stage, both EphA2 and EphrinA-1 protein expressions were significantly associated with a shorter clinical outcome for patients with ESCC in the univariate analysis as shown in our study. However, only clinical stage, number of lymph node metastasis and EphA2 protein expression were still significantly associated with poorer overall survival when multivariate analysis was applied, indicating that EPHA2 is a good prognostic marker for ESCC as reported (18).

The EphA2 receptor tyrosine kinase is overexpressed in a large number of human cancers. High levels of EphA2 expression have been observed in a large number of different cancers, including colon (10), breast, prostate and lung carcinomas, as well as metastatic melanomas (14-17). The highest levels of EphA2 are consistently found on the most aggressive cell models of human cancer (23-25). The fact that elevated EphA2 levels are found in multiple types of cancer suggests that EphA2 overexpression may be a common event in the metastatic progression of carcinoma cells (14).

EphrinA-1 and EphA2 were coordinately expressed in the tumour samples examined, suggesting that their expression may be regulated by similar factors or that overexpression of one protein induces the other. This is in accordance with other studies in which elevated levels of EphA2 have been shown to be related to poor prognosis and lymph node metastases in patients with oesophageal cancer (18) and lung cancer(16). For patients with malignant melanoma, overexpressions of both EphA2 and EphrinA-1 are associated with shorter survival (17). It has been reported that EphA2 is a powerful oncoprotein in breast cancer and that EphA2 overexpression causes malignant transformation (21). The results of our study support these observations.

In particular, the presence of lymph node metastases is associated with a poor prognosis in oesophageal cancer patients (26, 27). In fact, the number of diseased lymph nodes is the most important factor for the prognosis of squamous cell carcinoma of the oesophagus (28). EphA2 may be a good target to prevent ESCC cells from spreading into the lymphatic drainage. The intensity of EphA2 expression in preoperative biopsy specimens may be an indicator of advanced disease with a high probability of tumour spread. Further work is clearly required to investigate the relationship between EphA2 overexpression and tumour metastasis.

EphA2 overexpression causes defects in cell-cell contacts characteristic of aggressive cancer cells (14). EphA2 weakens cell-cell contacts and thereby prevents EphA2 from interacting with its ligands, which are anchored to the surface of neighbouring cells (14, 29, 30). Moreover, the EphA2 in these aggressive cancer cells is not tyrosine phosphorylated (29). One possible explanation for the weakened cell-cell adhesions is that overexpressed EphA2 may phosphorylate adhesion or cytoskeletal proteins and thereby destabilize cell-cell adhesions. Another possibility is that EphA2 alters the expression of important adhesion molecules. Future studies will be needed to identify the molecular targets of EphA2, such as cadherins and catenins, in ESCC.

EphrinA-1 ligand and the EphA2 receptor are usually co-localised in the same vascular endothelial and tumour cells, as reported in this study. Furthermore, statistical analysis indicates a significant association between EphA2 and EphrinA-1 expressions in ESCC. These findings suggest that EphA2 and Ephrin-A1 may play a role in tumour neovascularization. EphrinA1- is highly expressed in embryonic but not adult vasculature (31-33), suggesting a function in neovascularization but not in the stabilization of mature vessels. EphA2 expression has not been reported in embryonic or adult blood vessels, but EphA2 may participate in tumour neovascularization due to aberrant expression in tumour endothelial cells (20).

EphrinA-1 and EphA2 expressed in tumour cells may influence the interactions of endothelial cells with the surrounding tumour cells (20). It has been reported that EphrinA-1 ligand is mainly expressed in tumour cells and EphA2 receptor is localized primarily in tumour-associated vascular endothelial cells (34). Consistent with this, we did find EphA2 and EphrinA-1 proteins highly expressed in overlapping patterns in both tumour and endothelial cells in ESCCs. Taken together, these observations suggest that EphA2 and EphrinA-1 may play an important role in angiogenesis.

Although overexpression of EphA2 protein is detected in transformed mammary epithelial cells, EphA2 mRNA levels are equivalent in non-transformed and transformed mammary epithelial cell (35). To determine whether mRNA levels of EphA2 and EphrinA-1 corresponded with the protein levels, we collected about 200 tumour cells from each paraffin-embedded tissue section of 20 tumours by laser capture microdissection (LCM), and these cells were used for RNA extraction and RT-PCR. We found that mRNA expressions of EphA2 and EphrinA-1 did not fully correspond with their protein expressions. This discordance between mRNA and protein expression levels could be due to modifications at the post-transcriptional level and/or aberrant protein degradations of EphA2 and EphrinA-1 in ESCCs.

In conclusion, high levels of EphA2 expression are more often seen in tumour in men and are significantly associated with lymph node metastasis in ESCCs. Both EphA2 and EphrinA-1 overexpression is associated with unfavourable survival for ESCC patients in univariate analysis. In Cox multivariate analysis with clinical stage, histological grade, number of lymph node metastasis, EphA2 and EphrinA-1 included, only the number of lymph node metastases, clinical stage and EphA2 are strong independent predictors for poorer clinical outcome for ESCC patients. These results indicate that EphA2 may be a good target in preventing ESCC cells from spreading into the lymphatic drainage, supporting the notion that EphA2 is a good prognostic factor for her ESCC patients.

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