Low EphA7 Expression Correlated with Lymph Node Metastasis and Poor Prognosis of Patients with Esophageal Squamous Cell Carcinoma

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As a member of the Eph family of receptor tyrosine kinases, EphA7 plays an important role in cancer. However, the expression and significance of Eph receptors in esophageal squamous cell carcinoma (ESCC) remain unclear. Here, we detected the expression of EphA7 by immunohistochemistry in a sample of 352 patients with ESCC, and aimed to investigate the expression status of EphA7 in ESCC and its impact on prognosis. The results showed that low EphA7 expression significantly correlated with lymph node metastases (N0: 29%; N1: 64%. p<0.001), poor degree of tumor differentiation (G1: 31%; G2: 49%; G3: 58%. p=0.009) and pTNM staging (I+II: 33%; III+IV: 58%. p<0.001). Furthermore, in a combined analysis, patients with low EphA7-expressing tumors showed a shorter overall survival than those with high expression, resulting in a five-year overall survival rate of 47.4% vs. 52.6%, respectively (p=0.016). Consequently, patients with a low EphA7 expression have poorer prognosis in ESCC compared with those manifesting high expression.

I. Introduction

Esophageal squamous cell carcinoma (ESCC) is the most common malignant tumor of the upper aerodigestive tract [15]. The most difficult aspect of treating ESCC is its

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propensity for local invasion and metastasis, which is the leading cause of death in cancer patients [20]. Ephrin receptors (Ephs) named after the erythropoietin-producing hepatocellular carcinoma cell line from which their cDNA was derived, are over-expressed in numerous human tumors, with prognostic implications [24]. Eph receptors are the largest of receptor tyrosine kinases, and activated by binding of Ephrins [16, 17, 20]. According to their structural features and their preference for different Ephs, Eph receptors have been divided into 2 groups, designated EphA (A1–A8) and EphB (B1–B6). EphA receptors

preferentially bind to glycosylphosphatidylinositol (GPI)-anchored ligands such as ephrin-A (A1–A5), whereas EphB (B1–B6) receptors preferentially bind to transmembrane ligands such as ephrin-B (B1–B3) [6]. Those are phosphorylated after Eph/ephrin interaction [4]. The amino-terminal "Ephrin-binding" domain contains a high-affinity binding site that mediates receptor-Ephrin interaction between cells [9]. These activities depend on the Eph receptors. Ephrins activate complex bidirectional signaling networks and other signaling systems in physiology and disease [21, 23]. Eph receptors and ephrins are involved not only in early developmental processes, but also in adult physiology and the ability to modulate molecular signaling pathways is associated with important medical applications [10].

EphA7 (formerly known as Mdk1/Ebk/Ehk) is highly conserved in vertebrates from fish to humans [26]. It is widely expressed in embryonic tissues, especially in developing central nervous system [5]. Recent studies have demonstrated EphA7 expression in human breast cancer [8], prostate cancer [12], colorectal cancer [29], gastric carcinoma [30] and glioblastoma multiforme [31]. However, the relationship between EphA7 expression and tumor invasiveness, metastasis and prognosis in patients with ESCC is still unclear.

We conducted an immunohistochemical (IHC) analysis of EphA7 protein expression to determine the relationship between EphA7 expression and clinicopathological factors in ESCC.

II.   Materials and Methods

Patients and tissue specimens

ESCC specimens from 352 patients with primary esophageal cancer and adjacent normal esophageal epithelium from 36 of these patients, as well as another 17 cases of low-grade dysplasia tissues and 13 cases of high-grade dysplasia tissues from these patients, were obtained during surgical resection at the Department of Pathology of Shantou Central Hospital from 2003 to 2013. No patients had received radiotherapy or chemotherapy before the surgical resection. Of the 352 patients, 239 were male and 113 were female (ratio, 2.1: 1), with ages ranging from 37 to 81 years (median, 59 years). We used the revised TNM stage criteria by AJCC in 2009 [7]: stage I (n=33), II (n=161), III (n=158), IV (n=0). Evaluation of tumor differentiation was based on histological criteria of the World Health Organization guidelines [3]: complete (53 cases), moderate (270 cases) and poor differentiation (29 cases). All cases had complete follow-up records. The follow-up after esophageal resection was continued until death, and only patients who died of ESCC were included in the tumor-related deaths. Patients who had severe postoperative complications or other tumors, or those who died of other causes were excluded. This study was conducted under the regulations of the Institutional Review Board of Shantou Central Hospital. Informed consent was obtained from all the enrolled patients prior to surgery.

Tissue microarray (TMA) construction

TMA construction of esophageal carcinoma tissue has been described previously [33]. Briefly, TMA for immunohistochemistry were based on samples with adequate tissue available for persistent correlative studies. Representative tissue areas were demarcated from hematoxylin and eosin-stained sections on individual paraffin blocks. At least two tissue cores were acquired from each specimen, measuring 1.8 mm in diameter and 1.0 to 3.0 mm in length depending on the depth of tissue in the donor block. Each core was precisely arrayed into a new paraffin block. These microarrays were serially sectioned (4 μm) and stained with hematoxylin and eosin to ensure tissue sampling and completeness. The unstained sections were baked overnight at 56°C in preparation for immunohistochemistry staining.

Immunohistochemical analysis

The sections were dewaxed in xylene and rehydrated in a graded series of alcohols. Subsequently, slides were immersed in a peroxidase quenching solution containing one part of 30% hydrogen peroxide in nine parts of absolute methanol, for 10 min. After rinsing in PBS, antigen retrieval from the tissue was carried out by autoclaving in 0.01 M sodium citrate buffer (pH 6.0) at 120°C for 3 min. Next, sections were blocked in 10% normal goat serum for 10 min at room temperature and then incubated overnight at 4°C with rabbit anti-EphA7 monoclonal antibody (Abcam, Cambridge, UK; product number ab5400) against amino acids 26–41 of human Eph A7, at a dilution of 1:50. The sections were then subjected to immunostaining with the PV-9000 2-step plus Poly-HRP Anti-Mouse/Rabbit IgG Detection System (ZSGB-BIO, Beijing, China) and the Liquid DAB Substrate Kit (Invitrogen, San Francisco, CA). Samples were rinsed with distilled water. Subsequently, slides were counterstained with Mayer's Hematoxylin, dehydrated, and mounted.

Evaluation of immunostained samples

The immunostained samples were assigned a mean score based on the staining intensity and the proportion of tumor cells showing unequivocal positive reaction. Each section was independently assessed by two pathologists blinded to patients' data. Positive reactions were defined as those showing brown signals in the cell cytoplasm. For EphA7, a staining index (values 0–12) was determined by multiplying the staining intensity score with the positive reaction score. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The frequency of positive cells was graded as follows: 0, less than 5%; 1, 5% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, greater than 75%. Heterogeneous staining was scored for each component independently and the results were aggregated. For example, a specimen containing 75% tumor cells with
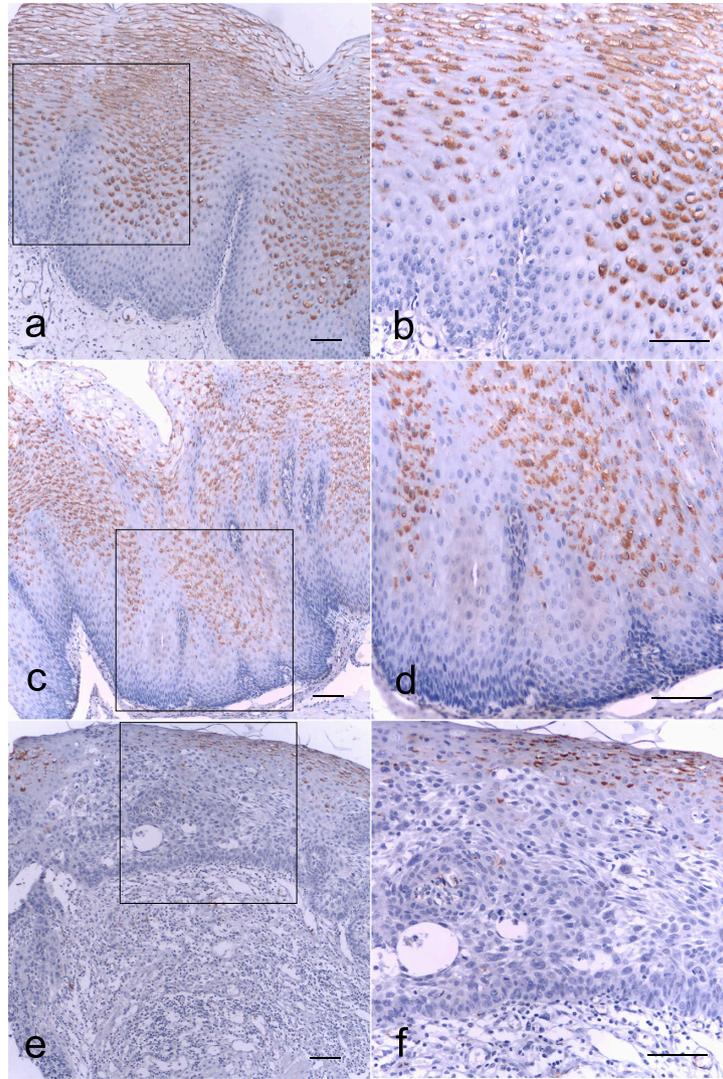
Fig. 1. Immunohistochemical analysis of EphA7 expression in the progression from normal esophageal mucosa (**a, b**), low-grade (**c, d**), to high-grade (**e, f**) dysplasia. EphA7 located in the cytoplasm and cell membrane of the differentiated zones (**a** and **b**). Immunostaining of EphA7 was also seen in the cytoplasm and cell membrane of low-grade (**c** and **d**) and high-grade dysplasia (**e** and **f**) but EphA7 was negative in the cytoplasm and cell membrane of high-grade dysplasia involving atypical cell layer (**e** and **f**). Bars=200 μm.

moderate intensity ($3 \times 2 = 6$), and another 25% tumor cells with weak intensity ($1 \times 1 = 1$) received a final score of $6 + 1 = 7$. For statistical analyses, scores of 0 to 7 were considered low expression whereas scores of 8 to 12 were deemed high [34].

Statistical analysis

SPSS 13.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. The relationship between EphA7 expression and other clinicopathological characteristics including age, gender, tumor size, tumor location, tumor differentiation grade, invasive depth, lymph nodes metastasis, and TNM stage, was analyzed using Pearson's Chi-Square test. Kaplan–Meier survival analysis (Log-Rank test) was used to evaluate differences in survival between patient subgroups. In addition, a Cox proportional hazards regression model was used to determine potential prognostic factors of postoperative survival. $P<0.05$ was considered statistically significant.

III. Results

Expression of EphA7 protein in normal esophageal tissue, esophageal dysplasia and ESCC

EphA7 expression in ESCC was investigated by immunohistochemical analysis of formalin-fixed, paraffin-embedded specimens using an EphA7-specific MAb. In normal esophageal tissue, EphA7 immunostaining appeared in the cytoplasm and cell membrane of the differentiated zones (Fig. 1a and b). Immunostaining of EphA7 was also seen in the cytoplasm and cell membrane of low-grade and high-grade dysplasia, particularly in cells located in the
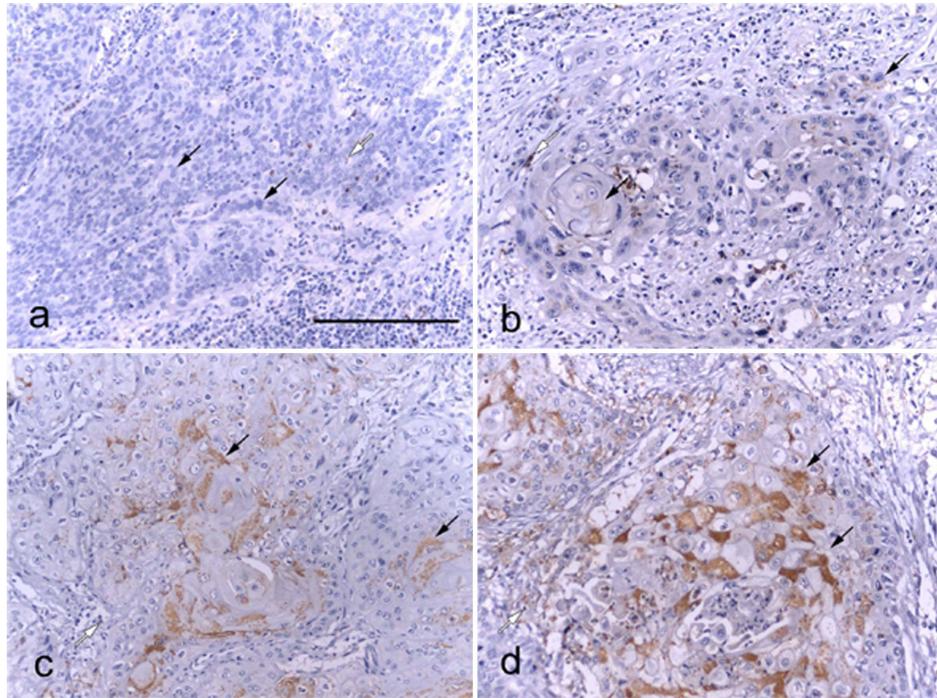
Fig. 2. Immunostaining analysis of EphA7 in ESCC. Representative photographs of EphA7 expression status. Black arrowheads show EphA7-positive cytoplasm in ESCC. White arrowheads show EphA7-positive regions in the cytoplasm of inflammatory cells. **a**, negative staining; **b**, weak staining; **c**, moderate staining; and **d**, strong staining. Bars=200 μm.

Table 1 *Association between A7 expression and parameters in esophageal squamous cell carcinoma*

Clinical Parameters		Low expression	High expression	X^2	p-value
Age	<59	88	94	0.019	0.749
	≥59	79	91		
Gender	Male	109	130	0.053	0.361
	Female	58	55		
Tumor size	≤3 cm	44	42		
	3–5 cm	70	90	0.002	0.985
	>5 cm	52	52		
Tumor location	Upper	7	14		
	Middle	68	78	0.059	0.266
	Lower	92	93		
Invasive depth	T1+T2	39	43	0.001	1.000
	T3+T4	128	142		
Differentiation	G1	16	35		
	G2	133	137	0.134	0.009
	G3	18	13		
Lymph node metastasis	N0	50	119	0.344	<0.001
	N1	117	66		
pTNM staging	I+II	63	129	0.321	<0.001
	III+IV	104	56		

Low expression (≤2), High expression (>2).

differentiated zones (Fig. 1c–f). However, the cytoplasm and cell membrane of the atypical cell layer cells in the high-grade dysplasia were EphA7-negative (Fig. 1e and f). In addition, weak, moderate or strong immunostaining of EphA7 was found in the cytoplasm of cancer cell nests with abundant keratin pearl cells (Fig. 2b–d). However, complete loss of EphA7 expression was found in the cytoplasm of cancer cell nests without formation of abundant keratin

pearl cells (Fig. 2a). These data indicate that EphA7 expression may be related to tumor differentiation.

EphA7 expression correlated with various clinicopathologic characteristics of ESCC

To elucidate the clinical significance of EphA7 expression in ESCC, we sought to determine possible correlation between EphA7 expression and various clinico-

Table 2 *Multivariate cox regression analysis of clinicopathologic factors for risk prediction in 352 patients with esophageal squamous cell carcinoma*

Factor	Risk	95% CI	p-value
Age	1.393	1.009–1.923	0.044
Gender	0.811	0.557–1.179	0.273
Tumor size	0.659	0.414–1.049	0.079
Tumor location	0.903	0.646–1.262	0.549
Invasive depth	1.101	0.700–1.734	0.677
Differentiation	0.547	0.335–0.894	0.016
pTNM staging	2.022	1.462–2.796	<0.001
Lymph node metastasis	2.113	1.519–2.940	<0.001
A7 high expression	0.874	0.613–1.248	0.459

pathologic characteristics of 352 ESCC cases (Table 1). Significant association between low cytoplasmic EphA7 expression and lymph node metastasis was observed (rs= 0.344; p<0.001). In the 183 cases of ESCC with lymph node metastasis, 66 patients (66/183; 36.1%) presented high cytoplasmic EphA7 expression, whereas 119 (119/169; 70.4%) of the 169 cases of ESCC without lymph node metastases exhibited high cytoplasmic EphA7 expression, suggesting that the ESCC cases without lymph node metastasis were more likely to show high cytoplasmic EphA7 expression. In addition, low expression of EphA7 was correlated with a poor degree of tumor differentiation (rs=0.134; p=0.009). Furthermore, in the 160 cases of ESCC with pTNM staging (III+IV), 56 cases (56/160; 35.0%) presented high cytoplasmic EphA7 expression, whereas 129 (129/192; 67.2%) of the 192 cases of ESCC with pTNM staging (I+II) exhibited high cytoplasmic EphA7 expression, suggesting that low EphA7 expression was correlated with TNM Classification (rs=0.321; p< 0.001). However, there was no significant association with age, gender, tumor size, tumor location or depth of tumor invasion.

Correlations between EphA7 expression and prognosis of patients with ESCC

The survival rate of patients with low EphA7 expression was significantly lower than in patients with high EphA7 expression (5-year survival rate 52.6% vs 47.4%, respectively; p=0.016; Fig. 3). These results indicate that EphA7 might be a potential prognostic marker of ESCC.

Further, a multivariate analysis of gender, age, tumor size, tumor location, invasive depth, differentiation, pTNM staging and lymph node metastasis was conducted. The results showed that EphA7 expression (risk=0.874; 95% CI≈0.613–1.248; p=0.459) was not an independent prognostic factor (Table 2).

IV. Discussion

ESCC is a lethal malignancy, and development of biomarkers for predicting prognosis is a clinical imperative. The Eph family of proteins is a potential candidate for pre-
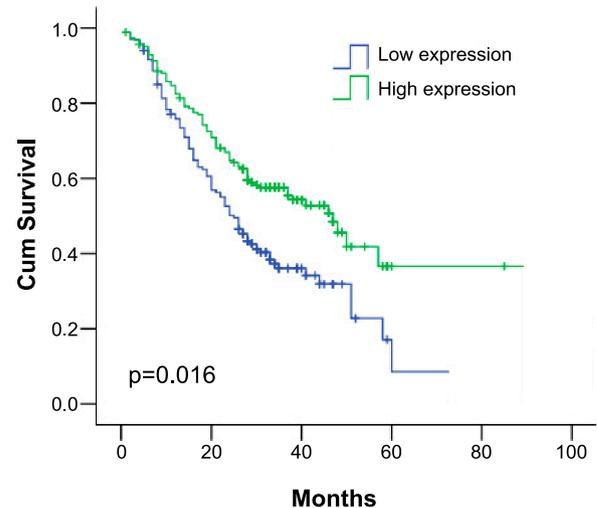

Fig. 3. Postoperative overall survival related to EphA7 expression. Patients with high EphA7 expression showed a significantly more favorable prognosis than those with low EphA7 expression (5-year survival rate: high expression, 44%; low expression, 56%; p=0.016).

dictive biomarkers. It has been reported that high EphA2 expression results in poor survival in ESCC [22]. EphA3, another member of Eph family, plays a tumor suppressor role in several tumors [28]. Previous studies have shown that EphA7 plays an important role in embryonic development of animals and humans [2, 25, 27]. Contrasting EphA7 levels of expression were observed in different tumors. For instance, the expression of EphA7 was upregulated in breast cancer [8] and gallbladder adenocarcinoma [19], but was downregulated in human gastric carcinoma, colorectal cancer and prostate cancer [12, 29, 30]. Therefore, EphA7 plays diverse roles in carcinogenesis. In our study, we first evaluated EphA7 expression in normal esophageal tissue and esophageal dysplasia by IHC analysis. We found that EphA7 was predominantly expressed in the cytoplasm and cell membrane of differentiated zones, whereas atypical cell layer cells did not show EphA7 immunoreactivity. Similar results were found in different tissues [13], but the Eph7 was immunostained in the cytoplasm of cancer cells, which was confirmed in our study.

Similar expression was also found in lung squamous cell carcinoma and adenocarcinoma [11]. We speculate that the result was attributed to truncated EphA7 protein expression in esophageal cancer. In addition, survival analysis showed that low EphA7 expression correlated with poor survival of ESCC.

Tumor metastasis is the main cause of death in most cancer patients. In particular, lymph node metastasis is associated with a poor prognosis in ESCC [1]. Previous studies demonstrated that high EphA7 expression was closely related to carcinogenesis, progression, clinical biological behaviors, and prognosis of glioblastoma multiforme [31] and gallbladder adenocarcinoma [19]. In contrast, we found that low expression of EphA7 protein was correlated with tumor differentiation, lymph node metastases and TNM Classification in ESCC. These results suggest that EphA7 may play a pivotal role in ESCC progression.

Previous studies suggested that EphA7 resembles other members of Eph family structurally, including a cysteine-rich region and tandem fibronectin type-III domains in extracellular portion of EphA7 [14]. Tyrosine phosphorylation of Eph/Eprin system promotes cellular transformation, invasion, proliferation, and also inhibits cellular spread or migration [18, 24, 32] mediated via JAK2, PI3K or ILK signal transduction pathways. However, downregulation of EphA7 resulting from methylation in human colorectal cancer leads to biological and histopathological effects associated with carcinogenesis and differentiation [12, 29]. These studies suggested that downregulation of EphA7 may also play an important role in carcinogenesis and differentiation of ESCC. However, specific signal transduction pathways of EphA7 mediating ESCC carcinogenesis have yet to be elucidated.

In conclusion, our results demonstrate that low EphA7 expression is involved in the differentiation and lymph node metastases of ESCC, suggesting that EphA7 may be associated with the progression of ESCC, and play an important role in the prognosis of ESCC patients.

V.   Acknowledgments

VI.   References

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