EphA8 is a Prognostic Factor for Oral Tongue Squamous Cell Carcinoma

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Background: Oral tongue squamous cell carcinoma (OTSCC) is the most common malignancy of the oral cavity. Here we explore the potential effects of EphA8, which is one of the receptors in Ephs subfamily of RTKs (receptor tyrosine kinases), in the progression and prognosis of OTSCC.

Material/Methods: A total of 119 OTSCC patients were enrolled in this retrospective study. Immunohistochemistry (IHC) staining and quantitative polymerase chain reaction (Q-PCR) were utilized to examine the expression of EphA8 in OTSSC tissues and adjacent non-tumor tissues. The relationship between EphA8 expression and the clinicopathological features of OTSCC patients were analyzed by chi-square. Survival analysis was carried out with Kaplan-Meier curve and the related log-rank test. Multivariate analysis was then undertaken to assess the prognosis factor by utilizing the Cox proportional hazard regression model. In addition, MTT assay and Matrigel invasion assay were performed to examine the effects of EphA8 on the proliferation and invasion capacities of human oral squamous carcinoma cells (SCC-25) and human tongue squamous cell carcinoma cells (H357).

Results: Q-PCR and IHC staining revealed that EphA8 was highly expressed in OTSCC tissues, especially in advanced stage OTSCC tissues. Kaplan-Meier curve showed that high EphA8 expression was significantly associated with poor prognosis, similar to age, smoking habit, drinking habit, tumor size, and TNM stage. Multivariate analysis indicated that EphA8 expression could serve as an independent prognostic factor in OTSCC. *In vitro* experiments revealed that overexpression of EphA8 might promote the progression of OTSCC via enhancing the invasion capacity but not proliferation capacity of tumor cells.

Conclusions: EphA8 was highly expressed in OTSCC tissues and was significantly associated with poor prognosis of OTSCC.

MeSH Keywords: **Mouth Neoplasms • Neoplasm Invasiveness • Prognosis**

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Background

Oral tongue squamous cell carcinoma (OTSCC) is one of the most common type of oral squamous cell carcinoma and is the sixth most common type of cancer worldwide [1]. It is a more aggressive disease than other sites of oral squamous cell carcinoma (OSCC) [2–4] and is always associated with poor prognosis, with nearly half of the patients dying [5]. The primary therapeutic strategy for advanced oral cancer is mainly surgery [6]; however, it inevitably causes many oral dysfunctions, like speech and swallowing dysfunctions. In recent years, radiotherapy with systemic chemotherapy has been used for advanced oral cancer, but this treatment outcome is still not considered to have satisfactory outcomes [7,8]. Of note, patients who were diagnosed at an early stage have a better prognosis [9]. In addition, the prediction of outcomes for OTSCC patients is essential for designing therapeutic strategies. These makes it of great importance to identify robust prognostic factors that could accurately predict the behavior of OTSCC.

Ephrin receptors (Ephs), which form the largest subfamily of receptor tyrosine kinases (RTKs), exhibit various functions by coupling with their ligand ephrins [10]. Ephs and ephrins are all membrane-anchor proteins and initiated bi-directional intracellular downstream signaling [11]. Ephs/ephrins signaling pathways have been shown to participate in many important developmental and cellular processes, like axon guidance [12] and angiogenesis [13,14]. Not surprisingly, it has been reported that Ephs are implicated in many aspects of cancer [14–16]. Due to their roles in angiogenesis, the Ephs system might enhance the growth capacity of tumors by increasing the vascularization process [14]. Meanwhile, the overexpressed Ephs in tumor tissues might also interrupt cellular adhesion, in turn, favoring the metastasis of tumors [16]. In addition, the Ephs system is also involved in regulating cell proliferation, apoptosis, invasion, and migration [17], indicating their essential roles in tumor progression.

EphA8 is one of the receptors in Ephs RTKs subfamily and functions through binding with GPI-anchored ephrin-A2, -A3, and -A5. A brain development study revealed that EphA8-Fc enhanced apoptotic cell death of the ephrin-A5 expressing cells in a caspase-dependent manner [18]. In addition, a cancer study reported that miR-10a/EphA8 pathway regulates epithelial-mesenchymal transition to affect cell migration and invasion [19]. Of note, EphA8 has been found highly expressed in epithelial ovarian cancer tissues compared to normal ovarian tissues; and high EphA8 protein level has been shown to be an independent prognostic factor in epithelial ovarian cancer [20]. However, so far, there is no study focusing on the role of EphA8 in the progression and prognosis of OTSCC.

In this current study, we enrolled 119 patients with TNM stage II or stage III OTSCC. EphA8 was found to be highly expressed in OTSCC tissues, especially in advanced OTSCC tissues. Univariate and multivariate analysis indicated that high EphA8 expression was significantly associated with poor prognosis and could serve as an independent prognostic factor in OTSCC. In terms of the effects of EphA8 expression on OTSCC, we performed a series of cellular experiments and found that overexpression of EphA8 promoted the progression of OTSCC via enhancing the invasion capacity but not the proliferation capacity of tumor cells.

Material and Methods

Patients and samples

The study protocol complied with the Helsinki Declaration and was approved by the Ethics Committee of Jinan Stomatological Hospital. From January 2009 to December 2016, a total of 119 patients who were diagnosed with OTSCC (TNM stage II or stage III) in Jinan Stomatological Hospital (Jinan, China) were enrolled in this retrospective study. The average age of the cohort enrolled was 54 years while the median age was 53 years. The follow-up period for patients varied from 18 months to 98 months while the median follow-up period was 61 months. By the end of the retrospective study, 54 enrolled patients were dead. The clinicopathological features are summarized in detail in Table 1.

Tumor stages of OTSCC patients were classified according to the TNM classification of the International Union against Cancer. The enrolled patients were diagnosed with either TNM stage II (62 cases) or stage III (57 cases). Paraffin-embedded sections were obtained from biopsy specimens of the enrolled 119 patients, with written informed consent from every patient enrolled in this study. In addition, fresh OTSCC tissues and adjacent non-tumor tissues were collected from another 17 cases of OTSCC patients and further applied to quantitative polymerase chain reaction (Q-PCR) test.

Immunohistochemical staining and evaluation

Levels of EphA8 in different OTSCC tissues were evaluated by immunohistochemical (IHC) staining. Serial 4-µm thick specimens were placed on silane-coated slides. Sections were deparaffinized in xylene and then incubated with antigen retrieve buffer at 121°C for 5 min. After that, endogenous peroxidase was blocked by incubating with 0.3% hydrogen peroxide. Sections were then blocked with 5% BSA for 1 hour at room temperature followed by incubating with the EphA8 primary antibody (1: 200, Cat# ab10615, Abcam, USA) at 4°C for overnight. Horseradish peroxide-conjunctive second antibody was applied to the sections the next day, incubating at 37°C for 30 min. DAB staining was then performed followed by counterstaining with hematoxylin [21].

Table 1. Correlations between EphA8 expression with patients' features.

Clinicopathologic features	Cases	EphA8 level		P value
	(n=119)	Low (n=51)	High (n=68)	
Age				0.368
≤54 yrs	62	29	33	
>54 yrs	57	22	35	
Gender				0.321
Female	43	21	22	
Male	76	30	46	
Smoking				0.540
No	41	16	25	
Yes	78	35	43	
Drinking				0.612
No	39	18	21	
Yes	80	33	47	
Tumor size				0.070
T1–T2	88	42	46	
T3–T4	31	9	22	
TNM stage				0.006*
II	62	34	28	
III	57	17	40	

EphA8 staining was evaluated and scored independently according to the following criteria by 2 pathologists who were double-blind to the clinical data. Levels of EphA8 in OTSCC tissues were determined by the degree of staining and the percent of positively stained cells. In brief, degree of staining was classified as no staining, weak staining, moderate staining, and strong staining and scored as 1, 2, 3, and 4 respectively. In terms of scoring the percentage of positively stained cells, 1 for less than 25%, 2 for 25% to 50%, 3 for 50% to 75%, and 4 for no less than 75%. Final scores were scaled from 1 to 16, which equaled the degree of staining score multiplied by the percentage of positively stained cells score. If the final score was no less than 8, it was regarded as high EphA8 expression case (68 cases); otherwise, it was counted as low EphA8 expression case (51 cases). The specific staining indicated that EphA8 mainly presented in the cytoplasm and cytomembrane of OTSCC cells.

Quantitative real-time PCR

Total RNA was first isolated from fresh OTSCC tissues and the adjacent non-tumor tissues by utilizing TRIzol reagent (Life Technology, CA, USA). The cDNA templates were then generated by performing reverse transcription PCR with isolated total RNA. Finally, real-time Q-PCR was performed to compare the expressions of EphA8 in OTSCC tissues and adjacent non-tumor tissues. GAPDH was chosen as an internal reference gene [22]. The primer sequences are as followed: EphA8 forward, 5'-CCACCAGGGTATGTAAATATC-3'; EphA8 reverse, 5'-TGTGCTTTGAAGACCATTT-3'; GAPDH forward, 5'-AGGGCTGCTTTTAACTCTGGT-3'; GAPDH reverse, 5'-CCCCACTTGATTTTGGAGGGA-3'.

Cell culture

Human oral squamous carcinoma cells (SCC-25) and human tongue squamous cell carcinoma cells (H357) were purchased from ATCC® and cultured in Dulbecco's Modified Eagle's Medium (DMEM): Ham's F12 Medium (1: 1) with 2 mM glutamine, 10% HyClone FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. SCC-25 and H357 cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Overexpression and knockdown of EphA8

EphA8 overexpression plasmid was generated by GenePharma. EphA8 siRNA was purchased from Santa Cruz Biotechnology. SCC-25 and H357 cells at about 70% confluence were

transfected with either EphA8 overexpression plasmid or EphA8 siRNA by using Lipo2000 for 24 hours before further experiments [23].

Western blot

Proteins were extracted from treated SCC-25 and H357 cells with lysis buffer. The same amount of proteins from each sample was applied to SDS-PAGE and immunoblotting analysis. Briefly, the membranes were incubated with anti-EphA8 and anti-GAPDH primary antibodies respectively at 4°C overnight. The membranes were then incubated with the HRP-conjugated secondary antibody at room temperature for 1 hour. Finally, electrogenerated chemiluminescence was utilized followed by exposing to x-ray films [24].

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The proliferation of SCC-25 and H357 cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following the manufacturer's procedure. In brief, SCC-25 and H357 cells were split into 96-well plates at a concentration of 5×10^3/well and further cultured for 24 hours. After transfected with either EphA8 overexpression plasmid or EphA8 siRNA for 0, 24, 48, 72, and 96 hours, SCC-25 and H357 cells were incubated with 0.5 mg/mL MTT in the dark for 4 hours. After that, the medium was replaced with dimethylsulfoxide (DMSO) and further shaken for 15 min [25]. Absorbance was recorded at 490 nm with Easy Reader 340 AT (SLT Labinstruments, Salzburg, Austria).

Invasion assay

The invasion capacities of SCC-25 and H357 cells were examined by Matrigel Transwell assay, as described by others [26]. Briefly, the upper chamber was first coated with diluted Matrigel coating (BD Biosciences, Franklin Lakes, NJ, USA). Then SCC-25 and H357 cells were seeded in the upper chamber at a density of 1×10^5 cells/well. After cells adhered, the medium in the upper chambers was changed to serum-free DMEM, and DMEM containing 10% FBS was added to the bottom chamber. After 48 hours, the non-invading cells were removed from the top side of the Transwell inserts, while the cells that passed through the Transwell inserts to the bottom side were fixed with 4% paraformaldehyde followed by staining for 5 min. The invading cells were photographed and counted in 5 high-power fields per insert. All experiments were conducted in triplicates.

Statistics

Statistical analysis was conducted by utilizing SPSS software (version 22; SPSS, Inc., Chicago, IL, USA). The relationships between EphA8 expression and the clinicopathological features of OTSCC patients was analyzed by chi-square (χ^2) tests. The differential expressions of EphA8 between the 2 groups were analyzed by Student's t-test. Survival analysis was carried out with Kaplan-Meier curve and the related log-rank test with factors including age, gender, smoking habit, drinking habit, tumor size, TNM stage, and EphA8 level. Multivariate analysis was then undertaken to assess the prognosis factor by utilizing the Cox proportional hazard regression model. P-value <0.05 was considered statistically significant.

Results

EphA8 expression in OTSCC tissues

In order to determine the potential effects of EphA8 expression in OTSCC onset and development, we first compared the expression levels of EphA8 between OTSCC tissues and adjacent non-tumor tissues. Fresh OTSCC tissues and adjacent non-tumor tissues were collected from 17 OTSCC patients and used for mRNA detection. Figure 1D shows EphA8 highly expressed in OTSCC tissues compared with the adjacent non-tumor tissues. Because the cohorts enrolled in our study were diagnosed with either TNM stage II or TNM stage III OTSCC, we further compared the EphA8 expression levels in different stages of OTSCC by performing IHC staining on specimens from 119 enrolled patients. Representative IHC staining showed that there was more EphA8 expression in stage III OTSCC tissues compared with stage II OTSCC tissues (Figure 1A, 1B). In addition, IHC staining was evaluated and scored according to the criteria described in our methods section. The IHC score of stage III OTSCC tissues was significantly higher than the IHC score of stage II OTSCC tissues (Figure 1C), indicating a potential role of EphA8 in the progression of OTSCC.

Relationship between EphA8 expression and clinicopathological features of OTSCC patients

Observing that EphA8 was highly expressed in OTSCC tissues, we further tried to analyze the relationships between EphA8 expression and clinicopathological features of OTSCC patients. A total of 119 TNM stage II and stage III patients enrolled in our study were divided into a high EphA8 expression group and a low EphA8 expression group according to the IHC staining score in which 8 scores was the boundary value. The clinicopathologic features of OTSCC patients enrolled in this study are shown in Table 1. We analyzed the relationships of EphA8 expression with age, gender, smoking habit, drinking habit, tumor size, and TNM stages respectively. Among them, TNM stages were significantly associated with EphA8 expression.
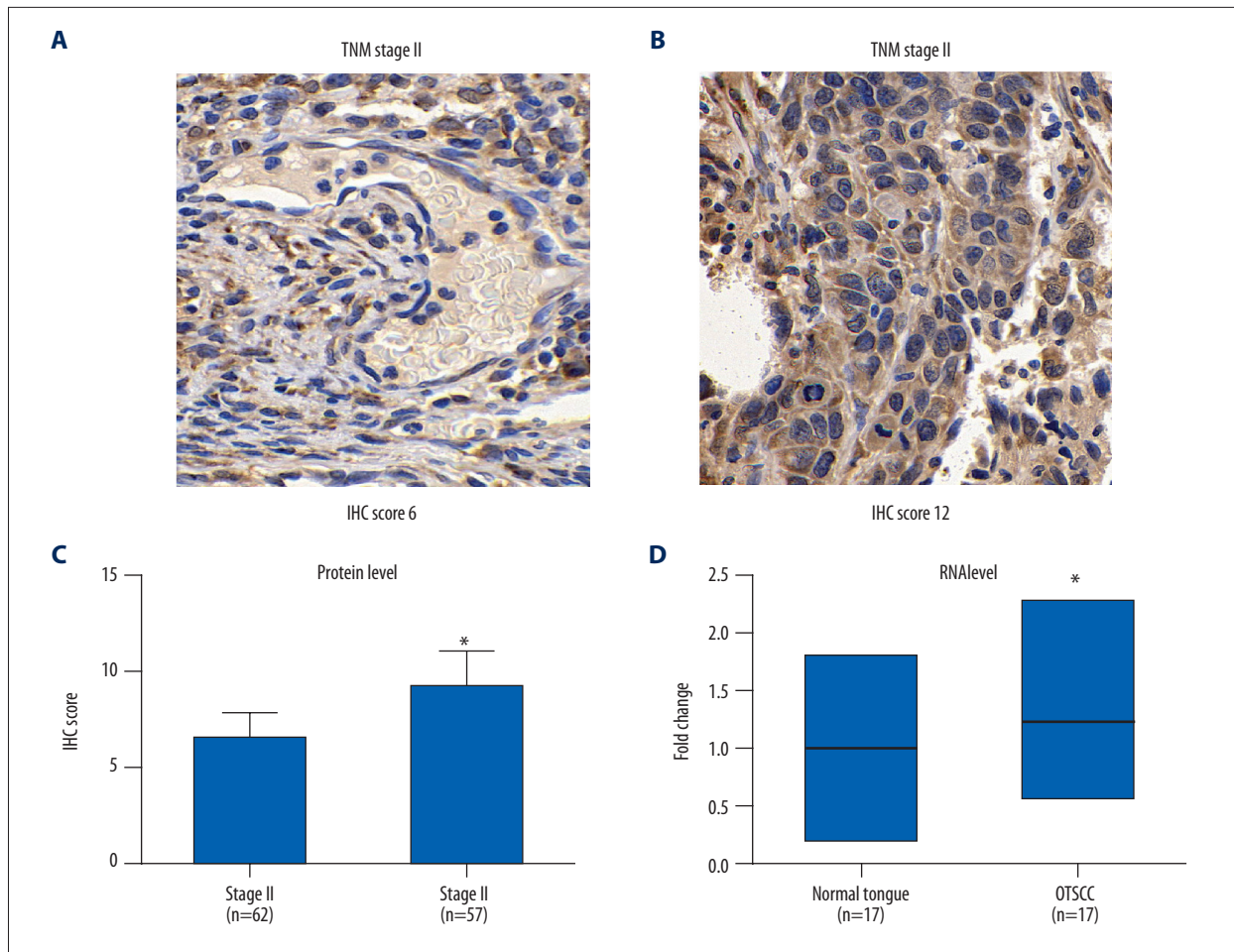
Figure 1. Expression of EphA8 in OTSCC tissues. (**A**) Representative immunohistochemical staining of EphA8 in TNM stage II OTSCC tissues, 400× magnification. (**B**) Representative immunohistochemical staining of EphA8 in TNM stage III OTSCC tissues, 400× magnification. (**C**) IHC staining against EphA8 in TNM stage II and III tissues were scored and used to perform the statistical analysis for EphA8 protein level. (**D**) The mRNA level of EphA8 in fresh OTSCC and adjacent non-tumor tissues were analyzed by Q-PCR. Data are mean ±SD from 3 independent experiments (* $P<0.05$). OTSCC – oral tongue squamous cell carcinoma.

Relationship between EphA8 expression and survival of OTSCC patients

Survival curve of the enrolled OTSCC cohort was plotted in Figure 2A. To further determine whether EphA8 expression is correlated with the overall survival of OTSCC patients, we utilized Kaplan-Meier curve and the related log-rank test to evaluate the survival of the 119 OTSCC patients enrolled in this study. Univariate analysis revealed that the prognosis of OTSCC patients could be predicted by age, smoking, drinking, tumor size, TNM stage, and EphA8 expression but not gender (Figure 2, Table 2). To be noted, OTSCC patients with high EphA8 expression presented a significantly poor prognosis with 61.5% compared to that of the low EphA8 expression group with 80.9% (Figure 2H), suggesting that high EphA8 expression was correlated with poor survival of OTSCC patients and might be a potential prognostic factor for OTSCC.

High EphA8 expression is an independent prognostic factor for survival of OTSCC patients

Due to the correlation of high EphA8 expression with a lower survival rate of OTSCC patients, we next explored whether high EphA8 expression could serve as an independent prognostic factor for OTSCC patients. We thus conducted the multivariate analysis by employing Cox proportional hazards regression model (Table 3). Multivariate analysis indicated that high expression of EphA8 (high *vs.* low; $P<0.05$) could serve as an independent prognostic factor, similar to tumor size (>5.0 cm *vs.* ≤5.0 cm; $P<0.05$). These results highly suggested that high EphA8 expression might be a potent predictor of OTSCC patients' survival.
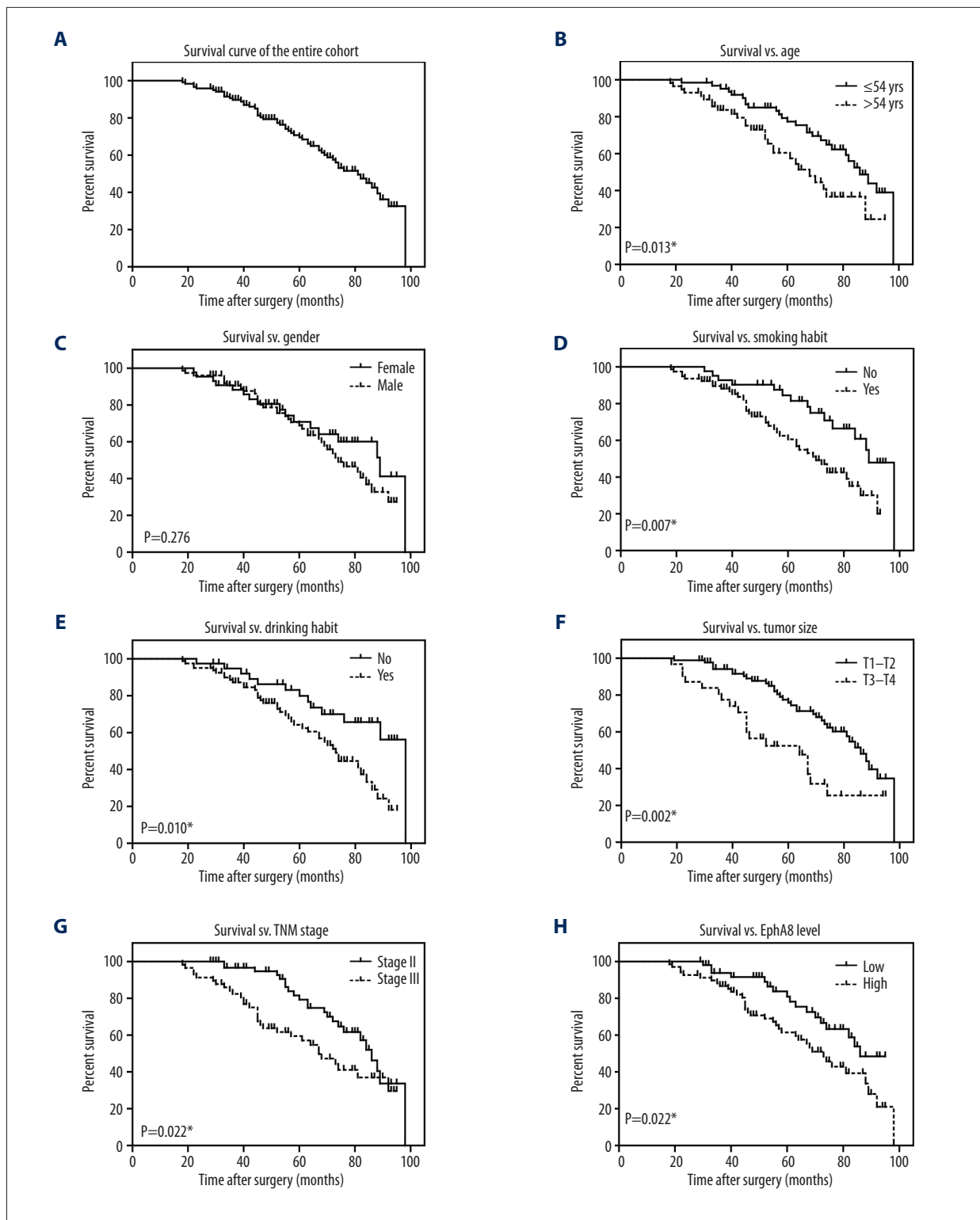
Figure 2. Kaplan-Meier analysis of overall survival. (**A**) Survival curve of the enrolled OTSCC cohort. Kaplan-Meier curve for tests of association between overall survival time and (**B**) age; (**C**) gender; (**D**) smoking habit; (**E**) drinking habit; (**F**) tumor size; (**G**) TNM stage; and (**H**) EphA8 level (* *P*<0.05). OTSCC – oral tongue squamous cell carcinoma.

Table 2. Kaplan-Meier overall survival analysis.

Clinicopathologic features	OS months (Mean ±S.D.)	5-year OS (%)	P value
Age			0.013*
≤54 yrs	79.2±3.0	77.3%	
>54 yrs	66.0±3.7	60.4%	
Gender			0.276
Female	76.4±4.4	70.8%	
Male	71.2±2.8	68.9%	
Smoking			0.007*
No	82.3±3.6	84.5%	
Yes	67.3±2.9	60.6%	
Drinking			0.010*
No	81.9±4.1	79.9%	
Yes	68.6±2.8	64.2%	
Tumor size			0.002*
T1–T2	78.2±2.6	75.9%	
T3–T4	59.8±4.9	52.4%	
TNM stage			0.022*
II	80.0±2.9	79.3%	
III	65.9±3.7	59.5%	
EphA8 level			0.022*
Low	79.3±3.1	80.9%	
High	68.6±3.3	61.5%	

Effects of EphA8 expression on the proliferation and invasion capacities of OTSCC cells

Tumor progression and metastasis are characterized by enhanced cell proliferation and invasion capacities. To determine the effect of EphA8 expression on the proliferation and invasion capacities of OTSCC cells, we performed MTT assay and Matrigel Transwell invasion assay. SCC-25 and H357 cells were transfected with either EphA8 overexpression plasmid or EphA8 siRNA. Transfection efficiency was validated by western blot analysis. As shown in Figure 3A and 3B, EphA8 was successfully overexpressed or knocked down in SCC-25 and H357 cells. Then we performed the MTT assay and the results indicated that EphA8 expression had no effect on the proliferation of SCC-25 and H357 cells (Figure 3C, 3D). Furthermore, we conducted the Matrigel Transwell invasion assay and found that overexpression of EphA8 could significantly enhance the invasion capacity of SCC-25 and H357 cells; and on the contrary, knockdown of

Table 3. Multivariate analysis.

Clinicopathologic features	HR	95% CI	P value
Age (>54 yrs *vs.* ≤54 yrs)	1.484	0.841–2.619	0.173
Smoking (yes *vs.* no)	1.888	0.956–3.728	0.067
Drinking (yes *vs.* no)	1.748	0.861–3.550	0.122
Tumor size (>5.0 cm *vs.* ≤5.0 cm)	2.449	1.143–5.248	0.021*
TNM stage (II *vs.* III)	0.915	0.436–1.921	0.815
EphA8 (high *vs.* low)	1.923	1.049–3.526	0.034*

EphA8 significantly reduced the invasion capacity of SCC-25 and H357 cells (Figure 3E, 3F). These results indicated that high EphA8 expression might promote the progression of OTSCC via enhancing the invasion but not the proliferation capacity of tumor cells, resulting in poor prognosis of OTSCC patients.

Discussion

As the most common malignancy of the oral cavity, OTSCC is an aggressive disease, with increasing incidence among young adults [27]. Due to the lack of robust early diagnostic markers, OTSCC patients are usually diagnosed at an advanced stage and have a poor prognosis. Although advances in therapeutic strategies for OTSCC have been developing, including surgery and radiotherapy with systemic chemotherapy, the treatment outcomes are still not satisfactory [7,8]. Thus, it is of great value for OTSCC treatment to identify robust markers for early diagnosis and prediction of prognosis.

Ephs are the largest subfamily of RTKs and have been showed to be implicated in many aspects of cancer [28,29]. In this study, we focused on one of the receptors in the Ephs RTKs subfamily, EphA8, which has been reported to be pivotal in epithelial ovarian cancer [20]. In order to figure out the potential effects of EphA8 in OTSCC, we enrolled a total of 119 OTSCC patients and performed a retrospective study. Patients only diagnosed as either TNM stage II or stage III OTSCC were enrolled in our study, for, on one hand, there is a very limited number of patients who were diagnosed as OTSCC at very early stage (stage I); on the other hand, patients at TNM stage IV have a very poor prognosis, making it hard to conduct a follow-up study. By examining the mRNA levels of EphA8 in fresh OTSCC tissues and adjacent non-tumor tissues, we found that EphA8 was highly expressed in OTSCC tissues comparing with non-tumor tissues, which was consistent with findings from an epithelial ovarian cancer study [20]. Furthermore, we also detected the differential expressions of EphA8 in different stages of OTSCC by performing IHC staining. EphA8 highly expressed in advanced
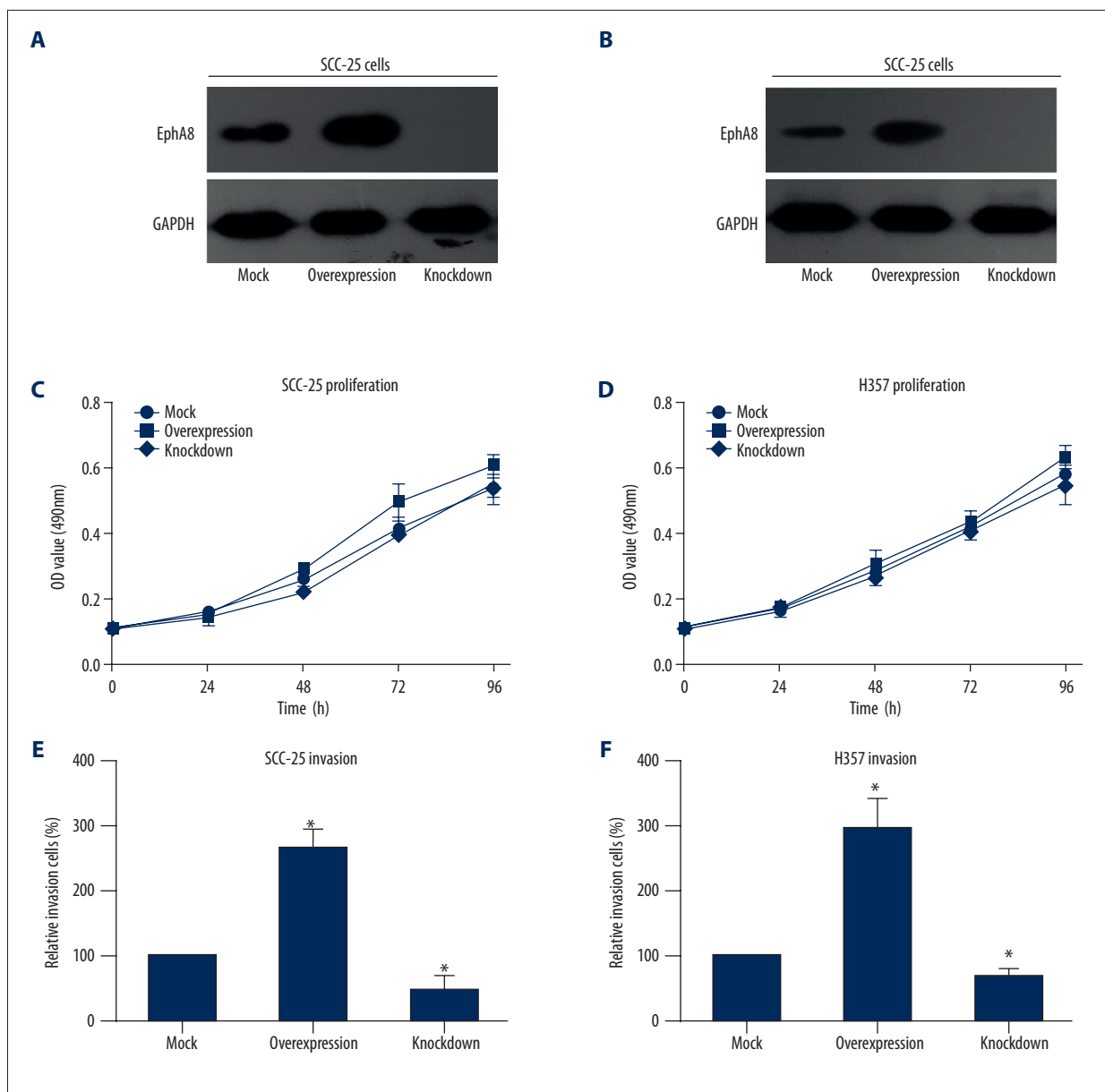
Figure 3. High EphA8 expression enhanced the invasion capacity but not proliferation capacity of SCC-25 and H357 cells. Transfection efficiencies of either EphA8 overexpression plasmid or EphA8 siRNA in SCC-25 (**A**) and H357 (**B**) cells were evaluated by detecting the protein levels of EphA8 with western blot analysis. MTT assay showed that expression of EphA8 had no effect on the proliferation capacity of SCC-25 (**C**) and H357 (**D**) cells. Matrigel Transwell invasion assay showed that overexpression of EphA8 enhanced the invasion of SCC-25 (**E**) and H357 (**F**) cells while knockdown of EphA8 inhibited the invasion of SCC-25 (**E**) and H357 (**F**) cells. Data are mean ±SD from 3 independent experiments (* *P*<0.05). SCC-25 – human oral squamous carcinoma cells; H357 – human tongue squamous cell carcinoma cells.

stage OTSCC patients comparing to early stage OTSCC patients. We then further analyzed the correlation of highly expressed EphA8 with the clinicopathological features and poor survival of OTSCC patients. Relationships of high EphA8 expression with some known risk factors for OTSCC were analyzed as well, including tobacco usage [30,31] and alcohol consumption [32]. We found EphA8 was only significantly correlated with TNM

stage, and not age, gender, smoking, drinking, or tumor size. These results indicated that EphA8 more likely participated in the progression of OTSCC.

As shown in previous studies [27,29], we also found that age, smoking habit, drinking habit, tumor size, and TNM stage were all significantly correlated with the overall survival of OTSCC

patients. Furthermore, we first found that high EphA8 expression was also significantly correlated with the overall survival of OTSCC patients; high EphA8 expression OTSCC patients presented a lower survival rate (61.5%) compared to patients with low EphA8 expression (80.9%).

Similar to tumor size, high EphA8 expression was also shown to be an independent prognostic factor for OTSCC by multivariate Cox proportional hazards regression model. Of note, EphA8 has been reported to be an independent prognostic factor in epithelial ovarian cancer [20]. It is thus of great value to explore whether EphA8 overexpression is a common process or mechanism in the progression of certain cancers.

As a membrane-anchored receptor, EphA8 exerts various functions via binding with its ligand ephrins. For example, EphA8-Fc enhanced apoptotic cell death of ephrinA5-expressing cells during brain development. In our study, we examined the possible effects of EphA8 expression on OTSCC via MTT assay and Matrigel invasion assay. We found EphA8 expression did not affect the proliferation of tumor cells. Consistent with other studies [19,33], we found high EphA8 expression could enhance the invasion capacity of tumor cells, which further supports that EphA8 participated in the progression of OTSCC. The exploration of the detailed underlying mechanisms will be of great value in drug development in the future.

Conclusions

In this current study, we found that EphA8 was highly expressed in OTSCC tissues and associated with OTSCC TNM stage. Univariate and multivariate analysis revealed that high EphA8 expression correlated with a poor survival rate of OTSCC patients and served as an independent prognostic factor for OTSCC. *In vitro* experiments support that EphA8 promoted the progression of OTSCC via positively regulating the invasion capacity of tumor cells.

Conflict of interest

None.

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