Pathol. Oncol. Res. (2014) 20:277–284
DOI 10.1007/s12253-013-9692-3

RESEARCH

Ephrin Receptor (Eph) -A1, -A2, -A4 and -A7 Expression in Mobile Tongue Squamous Cell Carcinoma: Associations with Clinicopathological Parameters and Patients Survival

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Received: 10 July 2012 / Accepted: 30 August 2013 / Published online: 11 September 2013

Abstract Ephrin receptors (Ephs) are frequently overexpressed in a wide variety of human malignant tumors, being associated with tumor growth, invasion, metastasis and angiogenesis. The present study aimed to evaluate the clinical significance of Eph-A1, -A2, -A4 and -A7 protein expression in mobile tongue squamous cell carcinoma (SCC). Eph-A1, -A2, -A4 and -A7 protein expression was assessed immunohistochemically on 37 mobile tongue SCC tissue samples and was analyzed in relation with clinicopathological characteristics, overall and disease-free patients' survival. All the examined mobile tongue SCC cases were found positive for Eph-A1, -A2, -A4 and -A7. Significant associations were noted between high Eph-A1, -A4 and -A7 expression and absence of lymph node metastases ($p=0.0263$, $p=0.0461$ and $p=0.0461$, respectively). High Eph-A1, -A2 and -A7 expression was significantly more frequently observed in patients presenting absence of vascular invasion ($p=0.0444$), dense stromal inflammatory reaction ($p=0.0063$) and female gender ($p=0.0327$), respectively. Mobile tongue SCC patients with high Eph-A7 expression presented longer overall and disease-free survival compared to those with low Eph-A7 expression (log-rank test, $p=0.0093$ and $p=0.0164$, respectively). In multivariate analysis, Eph-A7 expression was identified as independent prognostic factor of overall survival (Cox-regression analysis, $p=0.0426$). The present study supported evidence that Ephs may participate in the malignant transformation of mobile tongue SCC, reinforcing their utility as clinical markers for patients' management and prognosis, as also as targets for potential therapeutic intervention in tongue chemoprevention.

Keywords Mobile tongue squamous cell carcinoma · Ephrin receptors · Clinicopathological parameters · Prognosis · Immunohistochemistry

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Introduction

Ephrin (Eph) receptors constitute the largest sub-family of receptor tyrosine kinases, being divided into two sub-groups, EphA and EphB, based on their ligand-binding-affinity and structure of the extracellular domain (1, 2). Nine EphA (EphA1-9) and six EphB (EphB1-6) receptors have been identified to date. Their membrane-anchored ligands, the ephrins (ephs), are also divided into two sub-groups, ephA and ephB, which preferentially bind to EphA and EphB receptors, respectively (1–4). Ephs/ephs signaling has initially been shown to participate in a wide spectrum of developmental processes, being capable of regulating cellular adhesion, migration or chemo-repulsion and tissue/cell boundary formation (4, 5). Recent evidence has further extended the role of Eph receptors and their ligands as critical regulators of vascular remodelling during embryogenesis and tumor neovascularization. Thus, beyond their initial role, Ephs/ephs system has been involved in a broad range of processes directly related with tumorigenesis and metastasis, including cell attachment and shape, migration and angiogenesis (6–10). Moreover, unlike traditional oncogenes that often function

only in tumor cells, Eph receptors mediate cell to cell interactions both in tumor cells and tumor microenvironment, namely tumor stroma and vasculature, being considered as attractive targets for drug design (6–10).

Mobile tongue Squamous Cell Carcinoma (SCC) represents an aggressive malignancy with increasing incidence in Western communities which is frequently associated with poor prognosis (11, 12). Although new advanced therapeutic strategies have been applied to date, the 5-year survival rates have not been considerably improved (13, 14). This is mainly ascribed to the increased rate of lymph node metastasis, despite the fact that tumor diagnosis is currently been achieved at an early stage (15). The detection of occult metastasis remains difficult, so that the establishment and validation of prognostic markers in primary tumor specimens has been considered of high priority (16, 17).

Accumulative evidence has demonstrated that Eph receptors are overexpressed in a variety of tumors, being associated with important clinicopathological parameters for patients' management and prognosis (6–10, 18, 19). However, most of the available data so far is mainly restricted to Eph-A1 and -A2 receptors and concern, at a lower extent, other members of EphA family. Moreover, apart from the gradually increasing research in various malignancies, there is no comprehensive available data concerning the clinical significance of Ephs expression in mobile tongue SCC. In view of above considerations, the present study aimed to assess immunohistochemically Eph-A1, -A2, -A4 and -A7 expression in 37 mobile tongue SCC specimens, in association with clinicopathological parameters, as also overall and disease-free patients' survival.

Materials and Methods

Patients

Medical records and archival histopathological material of 37 mobile tongue SCC patients who were initially treated at Institut Curie, Paris, France, within the period 2000 to 2009 were included in this study. All patients underwent initial partial glossectomy as primary treatment, and 32 patients had elective neck dissections. The surgical specimens were examined at the Department of Pathology of the Institute Curie, according to standard histopathological protocols. Patients with prior radiotherapy, chemotherapy or surgery for malignancy were not included in the study.

Of the total 37 patients that were included in the study, 21 were male and 16 female with male to female ratio 1.31. The patients' age at first diagnosis ranged between 33 and 94 years (median age 60 years, IQR: 52–71 years). Clinical and histopathological parameters, including grade of histopathological differentiation, tumor thickness, nodal status, perineural invasion, and the presence of lymphovascular emboli were assessed (20). Tumor shape was classified as well and ill defined in case of either pushing or infiltrating tumor margins, respectively (21). The histological grading of tumor differentiation was based on conventional histological criteria included the assessment of keratinization, cellular and nuclear pleomorphism and mitotic activity, as well. Three typical scale grades were recorded: well, moderate and poorly differentiated (22). Tumor thickness was measured from the surface of the tumor to the deepest point of invasion (23, 24). Chronic inflammatory infiltration was identified as small mononuclear cells in the stroma of the entire tumor. The degree of infiltration was classified as mild, moderate and intense according to the density of inflammatory cells (20). Mitotic index in tumor cells was counted at X400 in ten consecutive randomly chosen fields using haematoxylin and eosin staining (24). The patients were followed-up from a time interval between 2 and 116 months (median 40 months, IQR: 12–65 months). Overall survival was defined as the time interval between the date of surgery and the date of death due to mobile tongue SCC. Disease free survival was defined as the time interval between the date of surgery and the date of detection of recurrence or the date of last follow-up without recurrence for mobile tongue SCC. At the time of the last follow-up, 12 (32.43 %) patients had died from disease, 2 (5.41 %) were alive with disease and 27 (62.16 %) were alive and disease-free.

Immunohistochemistry

Immunostainings for Eph-A1, -A2, -A4 and -A7 were performed on formalin-fixed, paraffin-embedded tissue sections using commercially available rabbit polyclonal Eph-A1 (S-20), Eph-A2 (H-120), Eph-A4 (H-77) and Eph-A7 (C-19) primary IgG antibodies (Santa Cruz Biochemicals, Santa Cruz, CA, USA). Briefly, 4 μm thick tissue sections were dewaxed in xylene and were brought to water through graded alcohols. Antigen retrieval (citrate buffer at pH 6.1 and microwave heating) was then performed. To remove the endogenous peroxidase activity, sections were then treated with freshly prepared 0.3 % hydrogen peroxide in methanol in the dark, for 30 min (min), at room temperature. Non-specific antibody binding was then blocked using Snipper, a specific blocking reagent for rabbit primary antibodies (Snipper, Biocare Medical, Walnut, Creek, CA, USA) for 5 min. The sections were then incubated for 1 h (h), at room temperature, with primary antibodies, diluted 1:100 in phosphate buffered saline (PBS). After washing three times with PBS, sections were incubated at room temperature with biotinylated linking reagent (Biocare Medical) for 10 min, followed by incubation with peroxidase-conjugated streptavidin label (Biocare Medical) for 10 min. The resultant immune peroxidase activity was

developed in 0.5 % 3,3′-diaminobenzidine hydrochloride (DAB; Sigma, Saint Louis, MO, USA) in PBS containing 0.03 % hydrogen peroxide for 5 min. Sections were counterstained with Harris' hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant anti-serum. As positive control, pancreatic and thyroid cancer tissue sections with known increased Eph positivity were used (18, 19).

Evaluation of Immunohistochemistry

Immunohistochemical evaluation was performed by counting at least 1,000 tumour cells in each case by two independent observers (S.T. and P.A.) blinded to the clinical data, with complete observer agreement. Specimens were considered "positive" for Eph-A1, -A2, -A4 and -A7 when more than 5 % of tumor cells within the section were positively stained (18, 19). The immunoreactivity of the tumor cells for Eph-A1, -A2, -A4 and -A7 was scored according to the percentage of Eph-A1, -A2, -A4 and -A7 positive tumor cells as 0: negative staining- 0–4 % of tumor cells positive; 1: 5–24 % of tumor cells positive; 2: 25–49 % of tumor cells positive; 3: 50–100 % of tumor cells positive, and its intensity as 0: negative staining, 1: mild staining; 2: intermediate staining; 3: intense staining. Finally, the expression of Eph-A1, -A2, -A4 and -A7 was classified as low; if the total score was 0 or 2 and high; if the total score was ≥3. In this way, we ensure that each group has a sufficient and more homogeneous number of cases in order to be comparable with the other groups (25, 26).

Statistical Analysis

Chi-square test was used to assess the associations of Eph-A1, -A2, -A4 and -A7 protein expression with clinicopathological variables. Survival curves were constructed using the Kaplan-Meier method and the differences between the curves were compared by the log rank test. A Cox proportional-hazard regression model was developed to evaluate the association between the potential prognostic marker and overall and disease-free survival. Cox regression analysis was conducted at both univariate and multivariate levels. A p-value less than 0.05 was considered the limit of statistical significance. SPSS for Windows Software was used for all analyses (SPSS Inc., 2003, Chicago, USA).

Results

All the examined mobile tongue SCC cases were found positive for Eph-A1, -A2, -A4 and -A7. All Eph receptors were abundantly and haphazardly expressed in the mobile tongue SCC cases examined, presenting mainly cytoplasmic and occasionally membraneous pattern of staining. Nuclear pattern of immunostaining was occasionally noted for Eph-A4 and -A7 (data not shown). Non neoplastic squamous tongue epithelium was found negative for Eph-A1, -A2 and -A4 and -A7. On the other hand, occasional cells in the basal layer of tongue squamous epithelium were found positive for Eph-A7. Eph-A1 expression was classified as high in 19 (51.35 %) out of 37 mobile tongue SCC cases. Eph-A2 expression was

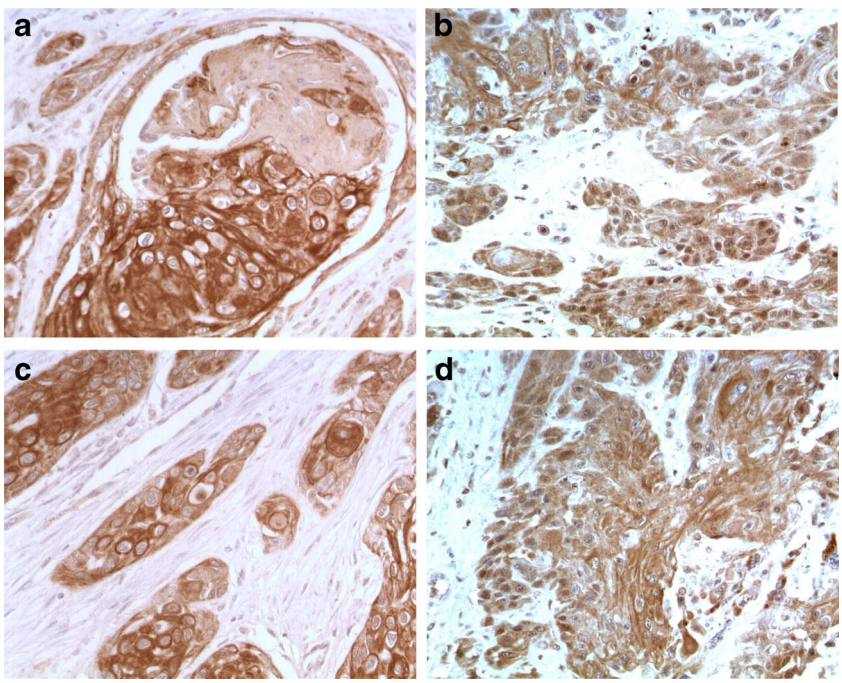

Fig. 1 Representative immunostainings for (**a**) Eph-A1, (**b**). Eph-A2, (**c**) Eph-A4 and (**d**) Eph-A7 protein expression in tumor cells of mobile tongue SCC. Streptavidin-biotin-peroxidase, DAB chromogen, Harris hematoxylin counterstain (original magnification X200)

classified as high in 16 (43.24 %) cases. Eph-A4 expression was classified as high in 19 (51.35 %) cases. Eph-A7 expression was classified as high in 18 (48.65 %) cases. Representative immunostainings for Eph-A1, -A2, -A4 and -A7 receptors are depicted in Fig. 1a–d, respectively.

High Eph-A1 expression was significantly associated with the absence of vascular invasion and lymph node metastasis (Table 1, $p=0.0444$ and $p=0.0263$, respectively). High Eph-A2 expression was significantly associated with dense stromal inflammatory reaction (Table 2, $p=0.0063$). High Eph-A4 expression was significantly associated with the absence of lymph node metastasis (Table 3, $p=0.0461$). High Eph-A7 expression was significantly more frequently observed in female patients compared to male ones, being also associated with the absence of lymph node metastasis (Table 4, $p=0.0327$ and $p=0.0461$, respectively). Trends of correlation between Eph-A7 expression and vascular invasion, as well as mitotic index were also noted (Table 4, $p=0.0633$ and $p=0.0696$, respectively). Eph-A1, -A2, -A4 and -A7 expression did not showed any significant or borderline associations

Table 1 Associations of Eph-A1 expression with clinicopathological parameters in 37 mobile tongue squamous cell carcinoma patients

Clinicopathological characteristics	Eph-A1 expression		
	Low	High	p-value
***N*=37**	18 (48.65)	19 (51.35)	
Age (mean ± SD;ys)			0.3847
≤60.83±14.32 years	12 (32.43)	10 (27.03)	
>60.83±14.32 years	6 (16.22)	9 (24.32)	
Gender			0.4193
Male	9 (24.32)	12 (32.43)	
Female	9 (24.32)	7 (18.92)	
Histopathological grade			0.2354
I	11 (29.73)	15 (40.54)	
II	7 (18.92)	4 (10.81)	
Stromal inflammatory reaction			0.2354
Mild/Moderate	7 (18.92)	4 (10.81)	
Dense	11 (29.73)	15 (40.54)	
Muscular invasion			0.3347
Yes	14 (37.84)	17 (45.95)	
No	4 (10.81)	2 (5.41)	
Shape			0.8003
Diffuse	13 (35.14)	13 (35.14)	
Well defined	5 (13.51)	6 (16.22)	
Vascular invasion			0.0444
Yes	7 (18.92)	2 (5.41)	
No	11 (29.73)	17 (45.95)	
Perineural invasion			0.8979
Yes	7 (18.92)	7 (18.92)	
No	11 (29.73)	12 (32.43)	
Depth of invasion			0.4141
I + II	11 (29.73)	14 (37.84)	
III	7 (18.92)	5 (13.51)	
Lymph node metastases			0.0263
Yes	9 (24.32)	3 (8.11)	
No	9 (24.32)	16 (43.24)	
Mitotic index			0.2476
≤median value	11 (29.73)	8 (21.62)	
>median value	7 (18.92)	11 (29.73)	

Statistical significant p-values are depicted by bold characters

Table 2 Associations of Eph-A2 expression with clinicopathological parameters in 37 mobile tongue squamous cell carcinoma patients

Clinicopathological characteristics	Eph-A2 expression		
	Low	High	p-value
***N*=37**	21 (56.76)	16 (43.24)	
Age (mean ± SD;ys)			0.7423
≤60.83±14.32 years	12 (32.43)	10 (27.03)	
>60.83±14.32 years	9 (24.32)	6 (16.22)	
Gender			0.1633
Male	14 (37.84)	7 (18.92)	
Female	7 (18.92)	9 (24.32)	
Histopathological grade			0.3667
I	16 (43.24)	10 (27.03)	
II	5 (13.51)	6 (16.22)	
Stromal inflammatory reaction			0.0063
Mild/Moderate	10 (27.03)	1 (2.70)	
Dense	11 (29.73)	15 (40.54)	
Muscular invasion			0.5924
Yes	17 (45.95)	14 (37.84)	
No	4 (10.81)	2 (5.41)	
Shape			0.8598
Diffuse	15 (40.54)	11 (29.73)	
Well defined	6 (16.22)	5 (13.51)	
Vascular invasion			0.1433
Yes	7 (18.92)	2 (5.41)	
No	14 (37.84)	14 (37.84)	
Perineural invasion			0.9705
Yes	8 (21.62)	6 (16.22)	
No	13 (35.14)	10 (27.03)	
Depth of invasion			0.8933
I + II	14 (37.84)	11 (29.73)	
III	7 (18.92)	5 (13.51)	
Lymph node metastases			0.5654
Yes	6 (16.22)	6 (16.22)	
No	15 (40.54)	10 (27.03)	
Mitotic index			0.8858
≤median value	11 (29.73)	8 (21.62)	
>median value	10 (27.03)	8 (21.62)	

Statistical significant p-values are depicted by bold characters

Table 3 Associations of Eph-A4 expression with clinicopathological parameters in 37 mobile tongue squamous cell carcinoma patients

Clinicopathological characteristics	Eph-A4 expression		
	Low	High	p-value
***N*=37**	18 (48.65)	19 (51.35)	
Age (mean ± SD;ys)			0.2540
≤60.83±14.32 years	9 (24.32)	13 (35.14)	
>60.83±14.32 years	9 (24.32)	6 (16.22)	
Gender			0.8858
Male	10 (27.03)	11 (29.73)	
Female	8 (21.62)	8 (21.62)	
Histopathological grade			0.8003
I	13 (35.14)	13 (35.14)	
II	5 (13.51)	6 (16.22)	
Stromal inflammatory reaction			0.6406
Mild/Moderate	6 (16.22)	5 (13.51)	
Dense	12 (32.43)	14 (37.84)	
Muscular invasion			0.4122
Yes	16 (43.24)	15 (40.54)	
No	2 (5.41)	4 (10.81)	
Shape			0.2354
Diffuse	11 (29.73)	15 (40.54)	
Well defined	7 (18.92)	4 (10.81)	
Vascular invasion			0.6336
Yes	5 (13.51)	4 (10.81)	
No	13 (35.14)	15 (40.54)	
Perineural invasion			0.8979
Yes	7 (18.92)	7 (18.92)	
No	11 (29.73)	12 (32.43)	
Depth of invasion			0.4141
I + II	11 (29.73)	14 (37.84)	
III	7 (18.92)	5 (13.51)	
Lymph node metastases			0.0461
Yes	3 (8.11)	9 (24.32)	
No	15 (40.54)	10 (27.03)	
Mitotic index			0.2476
≤median value	11 (29.73)	8 (21.62)	
>median value	7 (18.92)	11 (29.73)	

Statistical significant p-values are depicted by bold characters

Table 4 Associations of Eph-A7 expression with clinicopathological parameters in 37 mobile tongue squamous cell carcinoma patients

Clinicopathological characteristics	Eph-A7 expression		
	Low	High	p-value
***N*=37**	19 (51.35)	18 (48.65)	
Age (mean ± SD;ys)			0.8421
≤60.83±14.32 years	11 (29.73)	11 (29.73)	
>60.83±14.32 years	8 (21.62)	7 (18.92)	
Gender			0.0327
Male	14 (37.84)	7 (18.92)	
Female	5 (13.51)	11 (29.73)	
Histopathological grade			0.6406
I	14 (37.84)	12 (32.43)	
II	5 (13.51)	6 (16.22)	
Stromal inflammatory reaction			0.8003
Mild/Moderate	6 (16.22)	5 (13.51)	
Dense	13 (35.14)	13 (35.14)	
Muscular invasion			0.0633
Yes	18 (48.65)	13 (35.14)	
No	1 (2.70)	5 (13.51)	
Shape			0.2354
Diffuse	15 (40.54)	11 (29.73)	
Well defined	4 (10.81)	7 (18.92)	
Vascular invasion			0.2906
Yes	6 (16.22)	3 (8.11)	
No	13 (35.14)	15 (40.54)	
Perineural invasion			0.2194
Yes	9 (24.32)	5 (13.51)	
No	10 (27.03)	13 (35.14)	
Depth of invasion			0.1965
I + II	11 (29.73)	14 (37.84)	
III	8 (21.62)	4 (10.81)	
Lymph node metastases			0.0461
Yes	9 (24.32)	3 (8.11)	
No	10 (27.03)	15 (40.54)	
Mitotic index			0.0696
≤median value	7 (18.92)	12 (32.43)	
>median value	12 (32.43)	6 (16.22)	

Statistical significant p-values are depicted by bold characters

with the other clinicopathological parameters examined (Tables 1, 2, 3 and 4).

Univariate analysis was performed to assess the strength of the association of each clinicopathological parameter, Eph-A1, -A2, -A4 and -A7 (high versus low) with overall and disease-free patients' survival. Eph-A7 expression was identified as significant prognostic factor of overall and disease-free patients' survival ($p=0.0032$ and $p=0.0087$, respectively). Eph-A1, -A2 and -A4 expression and the other clinicopathological parameters examined did not show significant association with overall and disease-free patients' survival ($p>0.05$, data not shown).

Kaplan-Meier survival curves indicated that mobile tongue SCC patients with high Eph-A7 expression presented significantly longer overall survival times compared to those presenting low Eph-A7 expression (Fig. 2a, log-rank test, $p=0.0093$). Kaplan-Meier survival curves further indicated that mobile tongue SCC patients with high Eph-A7 expression had significantly longer disease-free survival times compared to those presenting low Eph-A7 expression (Fig. 2b, log-rank

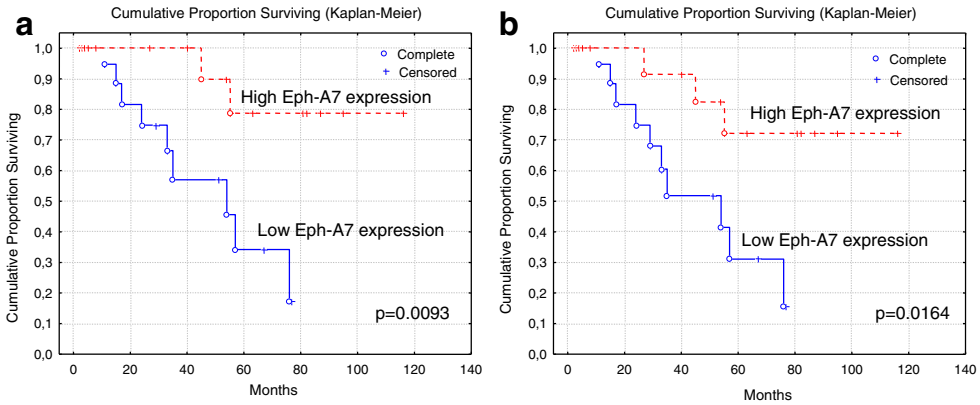

Fig. 2 Kaplan-Meier survival analysis stratified according to Eph-A7 expression in patients with mobile tongue SCC. **a** Overall survival and (**b**) Disease-free survival

test, $p = 0.0164$). In multivariate analysis, Eph-A7 expression was identified as independent prognostic factor of overall survival (Cox-regression analysis, $p = 0.0426$), whereas it did not remain significant in disease-free survival (Cox regression analysis, $p = 0.1432$).

Discussion

Accumulative evidence has suggested that Ephs and ephs are frequently overexpressed in a variety of human malignancies (5–10, 18, 19). However, the most comprehensive clinical data so far is mainly restricted to Eph-A1 and -A2 receptors, while the assessment of the clinical significance of Ephs in head and neck malignancies that focused on one anatomic site of the oral cavity, as in the case of mobile tongue remains still scarce.

Several previous studies have documented that Eph-A1 expression was associated with clinicopathological characteristic important for patients' management and prognosis in a variety of malignant tumors. Eph-A1 expression was associated with tumor size, histopathological stage and lymph node metastasis in pancreatic adenocarcinoma (18). In colorectal carcinomas, Eph-A1 expression was associated with patients' gender, histopathological grade and stage, depth of invasion, lymph node metastasis and survival (27, 28). Eph-A1 was overexpressed in urothelial carcinoma compared to normal tissues (29). A correlation between high Eph-A1 expression and high levels of cyclin A and p21, depth of invasion, advanced FIGO stage and poor patients' survival was reported in vulvar carcinomas (30). Hafner et al., also showed that Eph-A1 may represent a potential prognostic marker and therapeutic target in non-melanoma skin cancer (31). In this aspect, the present study further documented significant associations between high Eph-A1 expression and absence of vascular invasion and lymph node metastases, reinforcing its potential role in mobile tongue malignant disease progression.

Although the most comprehensive data so far demonstrated that Eph-A2 expression was associated with clinicopathological characteristic important for patients' management in a variety of malignant tumors (18, 19, 27–42), we did not find any clinical significance of Eph-A2 expression in mobile tongue SCC, except for a significant positive association with stromal inflammatory reaction. Notably, Eph-A2 overexpression was associated with poor prognosis in several types of malignant tumors, including that of oral tongue (28–30, 32–40). A recent study conducted on 59 oral tongue SCC showed that Eph-A2 and VEGF expression, as well as microvessel density (MVD) were correlated with tumor size, clinical stage, lymph invasion, recurrence and distant metastasis, being also identified as independent prognostic factors (36). However, our study did not show any prognostic effect of Eph-A2 expression on overall and disease-free patients' survival, which may be ascribed to the smaller number of cases examined.

As far as concern the less studied Eph members, Oki et al., reported that Eph-A4 overexpression was associated with the depth of invasion and the recurrence of gastric cancer patients (41). Eph-A4 overexpression was also associated with tumor proliferative capacity in pancreatic cancer patients (18). In this context, our study showed that high Eph-A4 expression was associated with the absence of lymph node metastasis and high Eph-A7 expression with longer overall and disease-free patients' survival. Eph-A7 expression was also associated with patients' gender and lymph node metastases, and borderline with muscular invasion and mitotic index. These observations support a potential role for Eph-A7 pathway signalling in the disease progression of mobile tongue SCC, including aspects that may affect patients' survival. Accordingly, Eph-A7 was reported to be predictive of the adverse outcome in recurrent glioblastoma multiforme (GBM) patients, being also identified as independent factor of poor prognosis in pancreatic ductal adenocarcinoma (18, 42).

In general, high Ephs expression has been documented to be correlated with an aggressive tumor phenotype and poor prognosis, whereas the present study revealed an inverse correlation. This controversy may be ascribed to the genomic context and the tissue and cell type-specific functions in the different types of cancer (43, 44). Ephs have been considered

as master regulators capable of either enhancing the activities of oncogenic signalling networks or repressing them, depending on ephs stimulation and other contextual factors (43, 44). Remarkably, Ephs and ephs can switch between contrasting activities by using bidirectional signalling, as well as other signalling modalities to influence cancer cell behaviour (43, 44). Taking into consideration the above notions, our findings supported evidence that Ephs may be considered as potential regulators capable of repressing the activities of oncogenic signalling in mobile tongue malignant disease progression, depending on ephs stimulation and/or other contextual factors.

Interestingly, accumulative *in vitro* and *in vivo* evidence suggested that Ephs/ephs signalling represent promising therapeutic target in cancer (43, 44). The functional cross-talk of Eph-A2 with other oncogenic alterations along in conjunction with encouraging results from pre-clinical combined studies with chemotherapeutic drugs or molecular therapies has reinforced the importance of combination therapies in targeting Ephs overexpression in cancer (44). Notably, Eph-A2 siRNA when used in combination with chemotherapeutic drug paclitaxel was more effective in inhibiting growth of ovarian tumors in mice than treatment with the control siRNA and paclitaxel (45). A combination of Eph-A2 and FAK siRNA resulted in a significant decrease in ovarian tumors and a reduction in tumor MVD compared to monotherapy (46). Eph-A2 overexpression was also identified as a contributing factor towards the development of resistance to Her2-targeted trastutzumab monoclonal antibody therapy (47).

Conclusions

The present study documented for the first time that Eph-A1, -A2, -A4 and -A7 were frequently overexpressed in human mobile tongue SCC, being associated with clinicopathological parameters crucial for patients' management and prognosis. Eph-A1, -A4 and -A7 were correlated negatively with lymphatic metastasis; however, only Eph-A7 remained an independent prognosticator for survival. These findings suggest an important potential role of Ephs, and especially Eph-A7 member, in mobile tongue malignant disease progression. Further research conducted on larger patients' cohort studies that additionally concern more sensitive techniques is strongly recommended. Understanding the complexity of Ephs participation in mobile tongue SCC could contribute to the elucidation of the mechanisms underlining cancer progression and metastasis that may in turn support the development of novel anti-cancer therapies targeting Ephs/ephs signalling system in this type of human malignancy.

Acknowledgment We thank Mrs Sofia Mastoraki and Mr Stylianos Georgiou for their excellent technical assistance.

Conflict of Interest Statement All authors verify that they have not accepted any funding or support from an organization that may in any way gain or lose financially from the results of the present study. All authors verify that they have not been employed by an organization that may in any way gain or lose financially from the results of the present study. None authors have any other conflicting interest.

References

1. Zhang J, Hughes SE (2006) Role of the ephrin and Ephrin receptor tyrosine kinase families in angiogenesis and development of the cardiovascular system. J Pathol 208:453–461
2. Pasquale EB (2008) Eph-ephrin bidirectional signaling in physiology and disease. Cell 133:38–52
3. Nakamoto M, Bergemann AD (2002) Diverse role for the Eph family of receptor tyrosine kinases in carcinogenesis. Microsc Res Tech 59: 58–67
4. Surawska H, Ma PC, Salgia R (2004) The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 15:419–433
5. Cheng N, Brantley DM, Chen J (2002) The ephrins and Eph receptors in angiogenesis. Cytokine Growth Factor Rev 13:75–85
6. Brandley-Sieders DM, Chen J (2007) Eph receptor tyrosine kinase in angiogenesis: from development to disease. Angiogenesis 7:17–28
7. Castaño J, Davalos V, Schwartz S Jr, Arango D (2008) EPH receptors in cancer. Histol Histopathol 23:1011–1023
8. Ireton RC, Chen J (2005) EphA2 receptor tyrosine kinase as a promising target for cancer therapeutics. Curr Cancer Drug Targets 5:149–157
9. Brantley-Sieders D, Schmidt S, Parker M, Chen J (2004) Eph receptor tyrosine kinases in tumor and tumor microenvironment. Curr Pharm Des 10:3431–3442
10. Heroult M, Schaffner F, Augustin HG (2004) Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. Exp Cell Res 312:642–650
11. Canto MT, Devesa SS (2002) Oral cavity and pharynx cancer incidence in the United States, 1975–1988. Oral Oncol 38:610–617
12. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ (2007) Cancer statistics. CA Cancer J Clin 57:43–66
13. Brenner H (2002) Long-term survival rates of cancer patients achieved by the end of the 20th century: a period analysis. Lancet 360:1131–1135
14. Shiboski CH, Schmidt BL, Jordan RC (2005) Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20–44 years. Cancer 103:1843–1849
15. Sano D, Myers JN (2007) Metastasis of squamous cell carcinoma of the oral tongue. Cancer Metastasis Rev 26:645–662
16. Kantola S, Parikka M, Jokinen K et al (2000) Prognostic factors in tongue cancer-relative importance of demographic, clinical and histopathological factors. Br J Cancer 83:614–619
17. Wangsa D, Ryott M, Avall-Lundquist E et al (2008) Ki-67 expression predicts locoregional recurrence in stage I oral tongue carcinoma. Br J Cancer 99:1121–1128
18. Giaginis C, Tsourouflis G, Zizi-Serbetzoglou A, Kouraklis G, Chatzopoulou E, Theocharis S (2010) Clinical significance of Ephrin (Eph)-A1, -A2, -A4, -A5 and -A7 receptors in pancreatic ductal adenocarcinoma. Pathol Oncol Res 16:267–276
19. Karidis NP, Giaginis C, Tsourouflis G, Alexandrou P, Delladetsima I, Theocharis S (2011) Eph-A2 and Eph-A4 expression in human benign and malignant thyroid lesions: an immunohistochemical study. Med Sci Monit 17:BR257–BR265
20. Brandwein-Gensler M, Teixeira MS, Lewis CM et al (2005) Oral squamous cell carcinoma: histological risk assessment, but not margin status, is strongly predictive of local disease-free and overall survival. Am J Surg Pathol 29:167–178

21. Shintani S, Matsura H, Hasegawa Y, Nakayama B, Fujimoto Y (1997) The relationship of shape of tumor invasion to depth of invasion and cervical lymph node metastasis in squamous cell carcinoma of the tongue. Oncology 54:463–467
22. Barnes L, Eveson JW, Reichert P, Sidransky D (2005) World Health Organization classifications tumours. Pathology and genetics of head and neck tumours. IARC Press, Lyon
23. Po Wing Yen A, Lam KY, Lam LK et al (2002) Prognostic factors clinically stage I and II oral tongue carcinoma: a comparative study of stage, thickness, shape, growth pattern, invasive front malignancy grading, Martinez-Gimeno score and pathologic features. Head Neck 24:513–520
24. Klijanienko J, el-Naggar AK, de Braud F (1995) Tumor vascularization, mitotic index, histopathologic grade, and DNA ploidy in the assessment of 114 head and neck squamous cell carcinomas. Cancer 75:1649–1656
25. Theocharis S, Klijanienko J, Giaginis C et al (2011) Histone deacetylase (HDAC)-1 and −2 expression in mobile tongue squamous cell carcinoma: associations with clinicopathological parameters and patients survival. J Oral Med Pathol 40:706–714
26. Theocharis S, Klijanienko J, Giaginis C et al (2011) Metallothionein expression in mobile tongue squamous cell carcinoma: associations with clinicopathological parameters and patients survival. Histopathology 59:514–525
27. Dong Y, Wang J, Sheng Z et al (2009) Downregulation of EphA1 in colorectal carcinomas correlates with invasion and metastasis. Mod Pathol 22:151–160
28. Saito T, Masuda N, Miyazaki T et al (2004) Expression of EphA2 and E-cadherin in colorectal cancer: correlation with cancer metastasis. Oncol Rep 11:605–611
29. Abraham S, Knapp DW, Cheng L et al (2006) Expression of EphA2 and Ephrin A-1 in carcinoma of the urinary bladder. Clin Cancer Res 12:353–360
30. Holm R, Knopp S, Suo Z, Tropè C, Nesland JM (2007) Expression of EphA2 and EphrinA-1 in vulvar carcinomas and its relation to prognosis. J Clin Pathol 60:1086–1091
31. Hafner C, Becker B, Landthaler M, Vogt T (2006) Expression profile of Eph receptors and ephrin ligands in human skin and downregulation of EphA1 in nonmelanoma skin cancer. Mod Pathol 19:1369–1377
32. Lin YG, Han LY, Kamat AA et al (2007) EphA2 overexpression is associated with angiogenesis in ovarian cancer. Cancer 109:332–340
33. Yuan W, Chen Z, Wu S et al (2009) Expression of EphA2 and E-cadherin in Gastric Cancer: correlated with Tumor Progression and Lymphogenous Metastasis. Pathol Oncol Res 15:473–478
34. Zeng G, Hu Z, Kinch MS et al (2003) High-level expression of EphA2 receptor tyrosine kinase in prostatic intraepithelial neoplasia. Am J Pathol 163:2271–2276
35. Kinch MS, Moore MB, Harpole DH (2003) Predictive value of the EphA2 receptor tyrosine kinase in lung cancer recurrence and survival. Clin Cancer Res 9:613–618
36. Shao Z, Zhang WF, Chen XM, Shang ZJ (2008) Expression of EphA2 and VEGF in squamous cell carcinoma of the tongue: correlation with the angiogenesis and clinical outcome. Oral Oncol 44:1110–1117
37. Kamat AA, Coffey D, Merritt WM et al (2009) EphA2 overexpression is associated with lack of hormone receptor expression and poor outcome in endometrial cancer. Cancer 115:2684–2692
38. Miyazaki T, Kato H, Fukuchi M, Nakajima M, Kuwano H (2003) EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. Int J Cancer 103:657–663
39. Wang LF, Fokas E, Bieker M et al (2008) Increased expression of EphA2 correlates with adverse outcome in primary and recurrent glioblastoma multiforme patients. Oncol Rep 19:151–156
40. Herrem CJ, Tatsumi T, Olson KS et al (2005) Expression of EphA2 is prognostic of disease-free interval and overall survival in surgically treated patients with renal cell carcinoma. Clin Cancer Res 11:226–231
41. Oki M, Yamamoto H, Taniguchi H, Adachi Y, Imai K, Shinomura Y (2008) Overexpression of the receptor tyrosine kinase EphA4 in human gastric cancers. World J Gastroenterol 14:5650–5656
42. Wang LF, Fokas E, Juricko J et al (2008) Increased expression of EphA7 correlates with adverse outcome in primary and recurrent glioblastoma multiforme patients. BMC Cancer 8:79
43. Pasquale EB (2010) Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer 10:165–180
44. Tandon M, Vemula SV, Mittal SK (2011) Emerging strategies for EphA2 receptor targeting for cancer therapeutics. Expert Opin Ther Targets 15:31–51
45. Landen CN Jr, Chavez-Reyes A, Bucana C et al (2005) Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. Cancer Res 65:6910–6918
46. Shahzad MM, Lu C, Lee JW et al (2009) Dual targeting of EphA2 and FAK in ovarian carcinoma. Cancer Biol Ther 8:1027–1034
47. Zhuang G, Brantley-Sieders DM, Vaught D et al (2010) Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. Cancer Res 70:299–308