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RESEARCH

Clinical Significance of EphB4 and EphB6 Expression in Human Malignant and Benign Thyroid Lesions

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Abstract Ephrin receptors (Ephs) are frequently overexpressed in a wide variety of human malignant tumors, being associated with tumor growth, invasion, angiogenesis and metastasis. The present study aimed to evaluate the clinical significance of EphB4 and EphB6 protein expression in human malignant and benign thyroid lesions. EphB4 and EphB6 protein expression was assessed immunohistochemically on paraffin-embedded thyroid tissues obtained from 127 patients with benign (n=71) and malignant (n=56) thyroid lesions. Enhanced EphB4 and EphB6 expression was more frequently observed in malignant compared to benign thyroid lesions (p=0.0508 and p=0.0006, respectively). EphB4 and EphB6 expression also provided a distinct discrimination between papillary carcinoma and hyperplastic nodules (p=0.0302 and p=0.0013, respectively). In malignant thyroid lesions, enhanced EphB4 expression was significantly associated with larger tumor size (p=0.0366). Enhanced EphB6 expression was significantly associated with larger tumor size (p=0.0366), the presence of lymph node metastases (p=0.0023), the presence of capsular (p=0.0038), lymphatic (p=0.0053) and vascular invasion (p=0.0018) and increased risk of recurrence rate (p=0.0038). The present study supported evidence that EphB4 and mainly EphB6 may participate in the malignant thyroid transformation, reinforcing their utility as useful biomarkers and possible therapeutic targets in this type of neoplasia.

Keywords Ephrin receptors · Thyroid malignancy · Clinicopathological parameters · Immunohistochemistry · Papillary carcinoma · Hyperplastic nodule

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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 (EphA1-8 and EphA10) and five EphB (EphB1-4 and Eph6) receptors have been identified to date. Their membrane-anchored ligands, the ephrins, are also divided into two sub-groups, ephrins-A and ephrins-B, which preferentially bind to EphA and EphB receptors, respectively [1–4]. Ephs and ephrins have been shown to form a vital cell communication system capable of bi-directional signaling [1, 2]. Ephs/ephrins signaling has initially been considered to participate in a wide spectrum of developmental processes by regulating cellular adhesion, migration or chemo-repulsion and tissue/cell boundary formation [4, 5]. Eph receptors and their ligands have also been involved in a broad range of processes directly related with tumorigenesis and metastasis, including cell attachment and shape, migration and angiogenesis [6, 7]. Additionally, Eph receptors have been considered as attractive targets for drug design, as targeting these molecules could simultaneously inhibit several aspects of tumour progression [6, 7].

Benign and malignant thyroid lesions constitute the most common neoplasia of endocrine glands with growing rates during the last two decades [8, 9]. Papillary thyroid carcinoma is the most common amongst thyroid malignancies,

accounting for more than 80 % of all thyroid cancers, while together papillary and follicular thyroid represent approximately 90 % of all thyroid cancers [10]. Thyroid cancer generally has a favourable outcome; however, a significant proportion of patients ultimately die from the disease due to local recurrences and/or distant metastases [11, 12]. Thyroid-stimulating hormone levels, thyroid ultrasound and fine-needle aspiration biopsy (FNAB) are key clinical tests to guide patients' management [13]. In many cases, the pathologists confronted thyroid lesions in which the distinction between benign and malignant malignancy can be quite subtle with clinical consequences in treatment modalities [14]. Currently, the identification of molecular biomarkers, which contribute to the discrimination of benign from malignant thyroid tumours and the estimation of their aggressiveness and/or behaviour, represents a diagnostic challenge [15, 16].

Accumulative clinical evidence has demonstrated that Ephs are overexpressed in a variety of tumors, being associated with clinicopathological parameters crucial for patients' management and prognosis. The most comprehensive data so far is mainly restricted to EphA subfamily [6, 7, 17–20]. In the last few years, the clinical significance of EphB subfamily members has also been assessed in several human malignancies [21–26]. Notably, EphB4 was shown to play an important role in the progression of papillary thyroid carcinoma by stimulating cell migration, in vitro, supporting evidence that EphB4 may be a potential therapeutic target in this type of neoplasia [27]. EphB4 expression was significantly upregulated in papillary carcinoma patients; however, no data concerning its association with crucial clinicopathological parameters for patients' management and prognosis was reported [27]. Moreover, regarding the role of EphB6 member in human malignancies, the currently available data remains scarce [28, 29]. In view of above considerations, the present study aimed to evaluate the EphB4 and EphB6 immunohistochemical proteins' expression in patients with benign and malignant thyroid lesions and to correlate it with important clinicopathological characteristics.

Materials and Methods

Patients

The study group consists of 127 histologically confirmed thyroid surgical specimens from an equal number of patients who had undergone thyroid surgery for benign and malignant lesions. Institutional review board approval was obtained to use archived material for research purposes. Seventy-one benign (59 hyperplastic nodules and 12 Hashimoto thyroiditis) and 56 malignant (46 papillary and 10 follicular carcinomas) cases were included in the study. Each neoplasm was classified according to the WHO histological classification of thyroid tumors [30]. The risk of recurrence was estimated according to the American Thyroid Association (ATA) staging system [31]. None of the patients had received any kind of anti-cancer treatment prior to surgery and there was neither clinical history of head and neck irradiation nor of other cancer.

Immunohistochemistry

Immunostainings for EphB4 and EphB6 were performed on formalin-fixed, paraffin-embedded tissue sections using commercially available rabbit polyclonal EphB4 (H-200, sc-5536) and EphB6 (H-90, sc-25461) primary IgG antibodies (Santa Cruz Biochemicals, Santa Cruz, CA, USA) for 1 h, at a dilution 1:150 and 1:200, respectively, as previously described [17–20]. As positive control, breast cancer tissue sections with known increased EphB4 and EphB6 positivity were used. Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant anti-serum. The follicular cells' proliferative capacity was assessed by Ki-67 immunohistochemical expression, as previously described [17–20].

Evaluation of Immunohistochemistry

Immunohistochemical evaluation was performed by counting at least 1000 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 EphB4 and EphB6 when more than 5 % of tumour cells within the section were positively stained [17–20]. The immunoreactivity of the tumor cells for EphB4 and EphB6 was scored according to the percentage of EphB4 and EphB6 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 [17–20]. Finally, the expression of EphB4 and EphB6 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 [17–20]. Ki-67 immunoreactivity was classified according to the percentage of positively stained follicular cells exceeded the median percentage value into two categories (below and over mean value), as previously reported [17–20].

Statistical Analysis

Chi-square tests were used to assess the difference of EphB4 and EphB6 immunoreactivity between the diverse histopathological entities (e.g., malignant *vs* benign thyroid lesions,

papillary carcinoma *vs* hyperplastic nodules, papillary carcinoma *vs* Hashimoto thyroiditis, follicular carcinoma *vs* hyperplastic nodules, follicular carcinoma *vs* Hashimoto thyroiditis, papillary *vs* follicular carcinoma and hyperplastic nodules *vs* Hashimoto thyroiditis). Chi-square tests were also used assess the associations between EphB4 and EphB6 immunoreactivity and clinicopathological characteristics in the subgroup of patients with malignant thyroid lesions. The association between EphB4 and EphB6 immunoreactivity and patients' age was assessed by the nonparametric Mann-Witney *U* test. A 2-tailed $p<0.05$ was considered statistically significant. Statistical analyses were performed using the software package SPSS for Windows (version 11.0; SPSS Inc., Chicago, IL, USA).

Results

Clinical Significance of EphB4 Expression in Thyroid Malignancy

EphB4 positivity (IHC score>0) was noted in 58 (46 %) out of 127 thyroid lesions. Forty-seven (37 %) of the examined cases presented moderate/strong immunoreactivity for EphB4 protein (IHC score≥3). The pattern of EphB4 distribution was predominantly cytoplasmic and occasionally membraneous. Normal surrounding areas adjacent to tumour were found negative for EphB4. Representative EphB4 immunostainings for hyperplastic nodule and papillary carcinoma are depicted in Fig. 1a and b, respectively.

In cross-tables, EphB4 immunoreactivity was borderline different between benign and malignant thyroid lesions (Table 1, $p=0.0508$). Moderate/strong EphB4 expression was significantly more frequently observed in papillary carcinomas compared to hyperplastic nodules (Table 1, $p=0.0302$). Moderate/strong EphB4 expression was more frequently observed in Hashimoto thyroiditis compared to hyperplastic nodules, as well as in folicullar carcinomas compared to hyperplastic nodules, at a non significant level though ($p=0.0890$ and $p=0.1132$, respectively). Non significant intergroup differences were observed for all the remaining possible comparisons between the different histopathological entities (data not shown). A trend of correlation between moderate/strong EphB4 expression and increased follicular cells' proliferation rate was also noted (Table 1, $p=0.0854$).

In the subgroup of malignant thyroid lesions, moderate/strong EphB4 expression was noted in 26 (46 %) out of 56 cases, being significantly more frequently observed in cases with large tumor size (Table 2, $p=0.0366$). Moderate/strong EphB4 expression was more frequently observed in older patients, as well as in those with presence of vascular invasion and increased follicular cells' proliferation rate, at a non significant level though (Table 2, $p=0.1075$, $p=0.1147$ and $p=0.1103$, respectively). Moderate/strong EphB4 expression was also more frequently observed in cases with moderate/high risk of recurrence rate, at a non significant level though ($p=0.3508$).

Clinical Significance of EphB6 Expression in Thyroid Malignancy

EphB6 positivity (IHC score>0) was noted in 52 (41 %) out of 127 thyroid lesions. Thirty-nine (31 %) of the examined cases presented moderate/strong immunoreactivity for EphB6

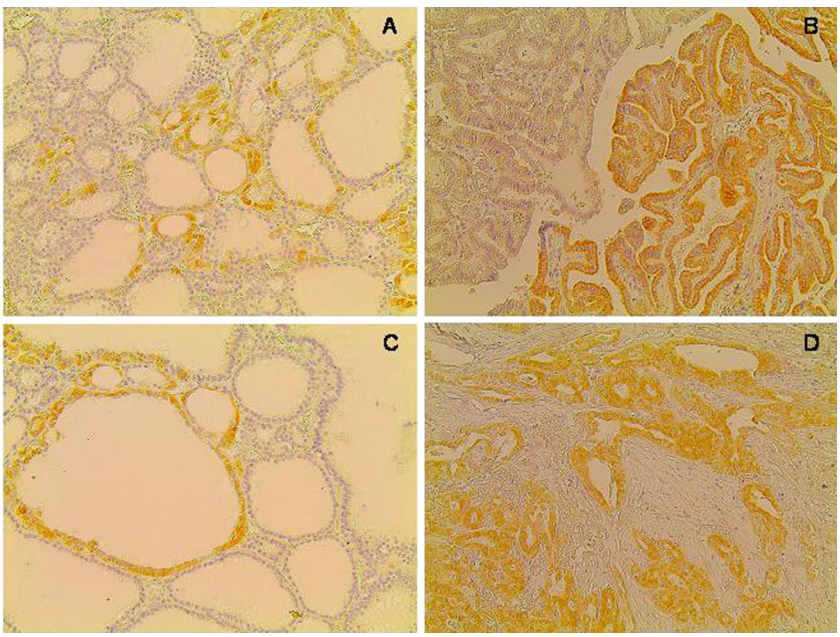

Fig. 1 Representative EphB4 immunostainings in: **a**. Hyperplastic nodule and **b**. Papillary carcinoma. Representative EphB6 immunostainings in: **c**. Hyperplastic nodule and **d**. Papillary carcinoma (original magnification X200)

Table 1 Associations of EphB4 and EphB6 expression with patients' age and gender, type of histopathology and Ki-67 protein statement in 127 patients with thyroid lesions

Clinicopathological characteristics	EphB4 expression			EphB6 expression		
	Negative/ Weak	Moderate/Strong	*p*-value	Negative/Weak	Moderate/Strong	*p*-value
N=127	80 (63 %)	47 (37 %)		88 (69 %)	39 (31 %)	
Age (mean±SD;yrs)	47.8±15.8	51.6±13.1	0.4584	48.8±15.1	50.1±14.8	0.6568
Gender			0.2528			0.5271
Female	64 (50 %)	41 (32 %)		74 (58 %)	31 (24 %)	
Male	16 (13 %)	6 (5 %)		14 (11 %)	8 (6 %)	
Histopathology (*N*=127)			0.0508			0.0006
Benign	50 (39 %)	21 (17 %)		58 (46 %)	13 (10 %)	
Malignant	30 (24 %)	26 (20 %)		30 (23 %)	26 (21 %)	
Histopathology (*N*=105)			0.0302			0.0013
Hyperplastic nodules	44 (42 %)	15 (14 %)		50 (48 %)	9 (9 %)	
Papillary carcinoma	25 (24 %)	21 (20 %)		26 (25 %)	20 (19 %)	
Ki-67 protein statement			0.0854			0.0395
< median value	38 (30 %)	15 (12 %)		42 (33 %)	11 (9 %)	
≥ median value	42 (33 %)	32 (25 %)		46 (36 %)	28 (22 %)	

protein (IHC score≥3). The pattern of EphB6 distribution was predominantly cytoplasmic and occasionally membraneous. Normal surrounding areas adjacent to tumour were found negative for EphB6. Representative EphB6 immunostainings

Table 2 Associations of EphB4 and EphB6 expression with clinicopathological characteristics in 56 patients with malignant thyroid lesions

Clinicopathological characteristics	EphB4 expression			EphB6 expression		
	Negative/ Weak (%)	Moderate/Strong (%)	*p*-value	Negative/ Weak (%)	Moderate/Strong (%)	*p*-value
N=56	30 (54 %)	26 (46 %)		30 (54 %)	26 (46 %)	
Age (mean±SD;yrs)	45.6±14.9	51.4±12.7	0.1075	46.8±14.1	49.9±14.8	0.3884
Gender			0.3998			0.5489
Female	24 (43 %)	23 (41 %)		26 (47 %)	21 (37 %)	
Male	6 (11 %)	3 (5 %)		4 (7 %)	5 (9 %)	
Tumor size (T)			0.0366			0.0366
T1	24 (43 %)	14 (25 %)		24 (43 %)	14 (25 %)	
T2-4	6 (11 %)	12 (21 %)		6 (11 %)	12 (21 %)	
Lymph node metastasis (N)			0.8394			0.0023
N0	26 (46 %)	23 (41 %)		30 (54 %)	19 (34 %)	
N1	4 (7 %)	3 (5 %)		0 (0 %)	7 (12 %)	
Capsular invasion			0.3508			0.0038
No	25 (45 %)	19 (34 %)		28 (50 %)	16 (28 %)	
Yes	5 (9 %)	7 (12 %)		2 (4 %)	10 (18 %)	
Lymphatic invasion			0.8965			0.0053
No	25 (45 %)	22 (39 %)		29 (52 %)	18 (32 %)	
Yes	5 (9 %)	4 (7 %)		1 (2 %)	8 (14 %)	
Vascular invasion			0.1147			0.0118
No	29 (52 %)	22 (39 %)		30 (54 %)	21 (37 %)	
Yes	1 (2 %)	4 (7 %)		0 (0 %)	5 (9 %)	
Ki-67 protein statement			0.1103			0.1103
< median value	13 (23 %)	6 (11 %)		13 (23 %)	16 (11 %)	
≥ median value	17 (30 %)	20 (36 %)		17 (31 %)	20 (35 %)	

for hyperplastic nodule and papillary carcinoma are depicted in Fig. 1c and d, respectively.

In cross-tables, EphB6 immunoreactivity was significantly different between benign and malignant thyroid lesions (Table 1, p=0.0006). Moderate/strong EphB6 expression was significantly more frequently observed in papillary carcinomas compared to hyperplastic nodules (Table 1, p=0.0013). A similar distinct discrimination between folicullar carcinomas and hyperplastic nodules was also obtained (p=0.0015). Moderate/strong EphB4 expression was more frequently observed in Hashimoto thyroiditis compared to hyperplastic nodules, at a non significant level though (p=0.1399). Non significant intergroup differences were observed for all the remaining possible comparisons between the different histopathological entities (data not shown). Moderate/strong EphB6 expression was also significantly associated with increased follicular cells' proliferation rate (Table 1, p=0.0395).

In the subgroup of malignant thyroid lesions, moderate/strong EphB6 expression was noted in 26 (46 %) out of 56 cases. Moderate/strong EphB6 expression was significantly more frequently observed in cases with large tumour size, presence of lymph node metastases and presence of capsular, lymphatic and vascular invasion (Table 2, p=0.0366, p=0.0023, p=0.0038, p=0.0053 and p=0.0118, respectively). Moderate/strong EphB6 expression was also significantly more frequently observed in cases with moderate/high risk of recurrence rate (p=0.0038).

Discussion

In the last few years, accumulative evidence has suggested that EphB receptors and their ligands are frequently overexpressed in a variety of human malignancies [21–26, 28, 29, 32–38]. However, to the best of our knowledge, there is no data so far concerning the clinical significance of EphB4 and EphB6 in human malignant and benign thyroid lesions.

In this aspect, this is the first study supporting clinical evidence that EphB4 and EphB6 are implicated in malignant thyroid transformation. The present study showed that EphB4 and EphB6 expression was increased in malignant compared to benign thyroid lesions, while a distinct discrimination between papillary carcinoma and hyperplastic nodule was also obtained. In malignant thyroid lesions, enhanced EphB4 expression was significantly associated with larger tumour size, while enhanced EphB6 expression with larger tumour size and the presence of lymph node metastases and capsular, lymphatic and vascular invasion and increased risk of recurrence estimated according to the ATA staging system. The above associations of EphB4 and EphB6 with clinicopathological parameters crucial for patients' management and prognosis supported evidence that EphB4 and EphB6 may be considered as useful biomarkers and possible therapeutic targets in thyroid malignancy.

In accordance with the present study, recent data suggested that EphB4 may play an important role in the progression of papillary thyroid carcinoma [27]. In fact, it was shown that EphB4 expression was significantly upregulated in papillary thyroid carcinoma patients [27]. Moreover, EphB4 overexpression in papillary thyroid carcinoma cell lines accelerated cell migration, while EphB4 downregulation inhibited cell migration, in vitro, and suppressed in vivo tumour metastasis [27]. Notably, EphB4 promoted cell migration by inhibiting the phosphorylation of FAK and paxillin, whereas EphB4 promoted cell migration in a kinase-independent manner [27]. Accordingly, in another study conducted by our group on EphA subfamily members, EphA2 was significantly overexpressed in malignant compared to benign thyroid lesions, while papillary carcinomas presented significantly increased EphA2 and EphA4 expression compared to hyperplastic nodules [19].

Among EphB subfamily, the most comprehensive clinical studies so far have been focused on EphB4 member. Notably, EphB4 overexpression was associated with advanced stage, smoking history and poor overall patients' survival in breast and head and neck squamous cell carcinoma (HNSCC) [34, 35]. EphB4 expression was increased according to the histopathological grade and Karnofsky performance scale (KPS) score, being also related to the progression-free survival of glioblastoma patients [32]. Enhanced EphB4 expression was associated with tumour aggressiveness based on clinical stage, lymph node metastasis, tumour size and poor prognosis in uterine cervical carcinoma [33]. EphB4 expression was correlated with tumour cell' differentiation, lymph node metastasis and TNM stage in NSCLC and HNSCC [34, 35]. In gastric cancer, significant associations between EphB4 overexpression and tumour size, as also presence of lymph node metastases was reported [23]. EphB4 expression in ovarian carcinoma was significantly associated with advanced disease stage, presence of ascites and poor patients' outcome [38]. A significant association between EphB4 expression and patients' survival in colorectal carcinoma was also reported [36]. In endometrial carcinoma, EphB4 expression was significantly associated with tumour histopathological grade and stage [37].

On the other hand, the existing clinical data so far concerning EphB6 subfamily member remains extremely scarce. More to the point, enhanced EphB6 expression was found in low- compared to advanced-stage tumors in neuroblastoma patients [39]. Moreover, the expression of TrkA, a well-established prognostic marker of favorable neuroblastoma, was positively correlated with EphB6 [39]. High EphB6 expression levels also predicted favorable neuroblastoma outcome [28]. Another study showed that melanoma progression to metastatic disease was associated with a significant reduction of EphB6 gene expression, which may have considerable

consequences for the prognosis of malignant melanoma patients, reinforcing possible gene-therapeutic approaches [29].

Currently, the Eph/ephrin system provides the foundation for potentially exciting new targets for anticancer therapies concerning Eph-expressing tumors. Accumulative in vitro and in vivo evidence has suggested that Ephs and their ligands may represent promising therapeutic targets in cancer, while a variety of strategies are currently under evaluation to interfere with their tumor-promoting effects or enhance their tumor-suppressing effects [40]. Notably, EphB4 inhibition reduced cellular viability, in vitro, halted the growth of established tumors in mouse xenograft models, in vivo, and caused near-complete regression of established tumours in combination with paclitaxel in lung cancer [41]. Specific small-molecule EphB4 inhibitor decreased cell viability in a time- and dose-dependent manner in esophageal cancer cell lines [42]. The above small-molecule inhibitor and an EphB4 siRNA also decreased cell migration, with decreased phosphorylation of various tyrosyl-containing proteins, including EphB4 and its downstream target p125FAK [42]. In a xenograft tumor model of esophageal cancer, EphB4 inhibition abrogated tumor growth by approximately 60 % compared to untreated control [42]. Accordingly, EphB4 targeting in ovarian cancer models resulted in reduced tumor angiogenesis, proliferation, and increased tumor cell apoptosis, which was ascribed to occur through modulation of phosphoinositide 3-kinase signaling [43].

Conclusions

The present study documented for the first time that EphB4 and EphB6 expression was increased in malignant compared to benign thyroid lesions. Distinct discrimination between papillary carcinoma and hyperplastic nodules and indicative discriminations between the other histopathological entities were obtained, dedicating the demand for larger cohort studies. In malignant thyroid subgroup, EphB4 and EphB6 expression was associated with clinicopathological parameters crucial for patients' management and prognosis. These findings suggest an important potential role of Ephs pathway signalling in thyroid malignant disease progression. However, further research effort is required to delineate whether EphB4 and EphB6 could be considered of diagnostic and prognostic utility and may exhibit targeted therapeutic potential in thyroid neoplasia.

Conflicts of interest 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.

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