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Evaluation of FAK and Src Expression in Human Benign and Malignant Thyroid Lesions

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Abstract Focal Adhesion Kinase (FAK) and Src have been reported to regulate tumor growth, invasion, metastasis and angiogenesis. The present study aimed to evaluate by immunohistochemistry the clinical significance of FAK and Src expression in 108 patients with benign and malignant thyroid lesions. Total FAK expression provided a distinct discrimination between malignant and benign (p=0.00001), as well as between papillary carcinoma and hyperplastic nodules thyroid lesions (p=0.00005), being also associated with follicular cells' proliferative capacity (p=0.0003). In malignant thyroid lesions, total FAK expression was associated with tumor size (p=0.0455), and presence of capsular (p=0.0102) and lymphatic (p=0.0173) invasion. Total Src expression was borderline increased in cases of papillary carcinoma compared to hyperplastic nodules (p=0.0993), being also correlated with tumor size (p=0.0169). FAK and Src expression was ascribed to a significant extent to the phosphorylated forms of the enzymes, which provided a better discrimination between malignant and benign thyroid lesions. The current data revealed that FAK and to a lesser extent Src expression could be considered of clinical utility in thyroid neoplasia with potential use as therapeutic targets.

Keywords Focal Adhesion Kinase (FAK) · Src · Thyroid cancer, immunohistochemistry · Clinicopathological parameters · Diagnosis

Christina Michailidi and Costas Giaginis contributed equally.

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Introduction

Focal adhesion kinase (FAK) is a 125 kDa cytoplasmic non-receptor tyrosine kinase enzyme initially described as a putative substrate for the Rous sarcoma virus-encoded oncoprotein $pp60^{v\text{-}src}$ [1, 2]. FAK was reported to be tyrosine-phosphorylated in response to integrin-mediated cell adhesion, integrin clustering, cell motility and migration [3, 4]. It was also shown that FAK forming a signaling complex with Src, a member of the Src family of cytoplasmic tyrosine kinases, activates downstream enzymes, such as mitogen-activated protein kinases (MAPK), resulting in activation of tumor cells [1, 3–5]. Src constitutes a non-receptor tyrosine kinase, encoded by the gene *c-Src*, while Src SH2 domain is available for binding with phosphotyrosine residues of other molecules, such as c-Met and FAK [6, 7]. The FAK/Src complex binds to and phosphorylates many downstream molecules, such as $p130^{Cas}$, Grb2 and PI3K, thereby transducing signals down different, complex cellular pathways, thus regulating various basic cellular functions, such as cell proliferation and growth, protection from apoptosis, adhesion, spreading, invasion and migration [8–11].

Both Src and FAK seem to play a crucial role in the malignant transformation and disease progression, while

accumulating in vitro and in vivo studies have identified FAK and Src as possible targets for anticancer therapy [12, 13]. Increased Src expression was reported in several human tumors including colorectal [14], pancreatic [15] and breast [16] cancer, while enhanced Src activity was implicated in the promotion of the malignant phenotypic characteristics of different cancer cell types [17–19]. Similarly, FAK upregulation was reported in various tumors, while unopposed FAK signalling appeared to promote tumor growth, progression, metastasis and angiogenesis. Currently, several in vivo studies have evaluated the diagnostic and prognostic significance of FAK expression in a variety of cancer types, such as liver, lung and cervical neoplasia underlining the crucial role of FAK in cancer biology [20].

Thyroid nodules represent the most common abnormality of endocrine glands, being diagnosed in 5% of the general population by palpation and in 50% by ultrasound [21]. During the last few decades, the incidence of thyroid cancer has gradually increased due to the increased detection of small papillary malignant lesions [22–24]. Although thyroid cancer generally presents a favorable outcome, a significant proportion of patients ultimately die from the disease due to development of local recurrence and/or distant metastasis [22–24]. Most thyroid tumors can be readily diagnosed using histopathological criteria, which allow the pathologist to differentiate malignant from benign lesions and to verify an accurate classification for the majority of the thyroid carcinoma variants [25]. However, in many cases, the pathologist is confronted with thyroid lesions in which the distinction between benign and malignant state can be quite subtle and the decision favoring one or another results in harmful clinical consequences and implies different treatment modalities [25]. In this context, western blot analysis on 30 patients showed that FAK expression was directly associated with the aggressive phenotype of thyroid carcinoma, as follicular type tumors presented enhanced FAK expression compared to malignant thyroid tissues with reduced invasive potential, such as papillary carcinomas, follicular adenomas, and other non malignant thyroid lesions [26]. A more recent study conducted on 47 patients documented by immunohistochemistry that FAK expression was significantly increased in malignant compared to benign thyroid lesions [27]. FAK was also shown to play a crucial role in promoting cell invasion through the activation of distinct signaling pathways induced by epidermal growth factor (EGF) with protein matrix metalloproteinase (MMP)-9 transcription and secretion in follicular thyroid carcinoma cells, supporting evidence that FAK may represent an important therapeutic target in thyroid neoplasia [28]. On the other hand, to our knowledge, there are no data so far concerning the clinical impact of Src in thyroid neoplasia.

In the light of the above considerations, the purpose of the current study was to examine the immunohistochemical expression of FAK and Src in 108 patients with benign and malignant thyroid lesions and to assess their diagnostic utility. We also aimed to evaluate the association of FAK and Src expression with important clinicopathological characteristics for the management of patients with malignant thyroid tumors, such as tumor size and lymph node metastases, as well as capsular, lymphatic and vascular invasion.

Patients and Methods

Patients

One hundred and eight formalin-fixed, paraffin-embedded thyroid tissues from an equal number of patients who had undergone thyroid surgery for benign or malignant disease were included in this study. None of the patients received any kind of anti-cancer treatment prior to surgery. The mean age of the patient cohort was 52.35±14.37 years (range: 20–72 years). Each case was classified according to the WHO histological classification of thyroid tumors [29]. The clinical material consists of 48 benign (39 hyperplastic nodules and 9 Hashimoto thyroiditis) and 60 malignant (45 papillary, 8 medullary, 5 follicular and 2 anaplastic thyroid carcinomas) cases. The characteristics of the study population stratified by benign and malignant histopathology are summarized in Table 1.

Immunohistochemistry

FAK and Src immunostaining was performed on formalin-fixed, paraffin-embedded thyroid tissue sections using a mouse anti-human FAK IgG_1 antibody, raised against the COOH-terminal of total FAK (t-FAK) protein (sc-1688, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and another mouse anti-human total c-Src (t-Src) IgG_{2a} antibody (sc-5266, Santa Cruz Biotechnology), respectively. A rabbit polyclonal anti-phospho FAK (Tyr 861, Stressgen Corporation Bioreagents, Glanford Ave, Victoria, Canada) and another rabbit polyclonal anti-phospho Src (Tyr 418, Stressgen Corporation Bioreagents) were used to assess the phosphorylated forms of FAK (p-FAK) and Src (p-Src) proteins, respectively. Briefly, 4 μm thick tissue sections were dewaxed in xylene and were brought to water through graded alcohols. To remove the endogenous peroxidase activity, sections were 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 blocked using Snipper, a specific blocking reagent for mouse and rabbit primary antibodies (Sniper, Biocare

Table 1 Study population characteristics

	Benign	Malignant
***N*=108**	48 (44.44%)	60 (55.56%)
Age (mean±SD;ys) 52.35±14.37 (range: 20–72)	53.79±15.10	51.20±13.78
Gender		
Male	10 (9.26)	10 (9.26)
Female	38 (35.19)	50 (46.30)
Histopathology	Hyperplastic nodule 39 (36.11)	Papillary 45 (41.67)
	Hashimoto's thyroiditis 9 (8.33)	Medullary 8 (7.41)
		Follicular 5 (4.63)
		Anaplastic 2 (1.85)
Tumor size (T)	N/A[a]	
T1		42 (70.00)[b]
T2		13 (21.67)[b]
T3		2 (3.33)[b]
T4		3 (5.00)[b]
Lymph node metastases (N)	N/A	
N0		53 (88.33)[b]
N1		7 (11.67)[b]
Distant metastases (M)	N/A	
M0		60 (100)[b]
Capsule invasion	N/A	
No		52 (86.67)[b]
Yes		8 (13.33)[b]
Lymphatic invasion		
No		53 (88.33)[b]
Yes		7 (11.67)[b]
Vascular invasion	N/A	
No		56 (93.33)[b]
Yes		4 (6.67)[b]
Ki-67 protein statement		
< mean value	41 (37.96)	31 (28.70)
≥ mean value	7 (6.48)	29 (26.85)

[a] N/A: not applicable

[b] Percentages in parentheses correspond to the number of malignant thyroid cases

Medical, Walnut, Creek, CA, USA) for 5 min. The sections were incubated for 1 h (h), at room temperature, with the primary antibodies against t-FAK, t-Src, p-FAK and p-Src, diluted 1:80, 1:200, 1:100 and 1:300, respectively, 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 with DAB substrate kit (Vector Laboratories, USA) for 10 min. Sections were counterstained with Harris' hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). An additional step of antigen retrieval (citrate buffer at pH 6.1 and microwave heating) was performed before incubation with the primary antibodies. Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant anti-serum. Gastric cancer tissue sections with known increased FAK and Src positive immunoreactivity were used as positive control [30]. The tumor proliferative capacity was assessed immunohistochemically, using a mouse *anti*-human Ki-67 antigen; IgG_{1k} antibody (clone MIB-1, Dakopatts, Glostrup, Denmark) as previously described [30–32].

Evaluation of Immunohistochemistry

The immunohistochemical evaluation was performed by counting at least 1,000 follicular 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 total and phosphorylated FAK

and Src when more than 5% of the follicular cells were stained [30–32]. The immunoreactivity of the follicular cells for Ki-67 protein antigen was classified according to the percentage of positively stained cells exceeded the mean percentage value into two categories (below and over mean value), as previously reported [30–32].

Statistical Analysis

The associations of t-FAK and t-Src positivity with clinicopathological characteristics were assessed by chi-square test for categorical variables and Mann-Whitney *U* test for continuous variables (age). Chi-square test was also used to compare t-FAK and t-Src positivity between malignant and benign thyroid lesions, as well as papillary carcinoma cases and hyperplastic nodules, which comprise the most numerous histopathological entities of malignant and benign cases, respectively. Mann-Whitney U and Kolmogorov-Smirnov tests were further used to evaluate the difference of the extent of t-FAK and t-Src immunoreactivity, defined as the percentage of t-FAK or t-Src - positive follicular cells, between malignant and benign thyroid lesions, as well as between papillary carcinoma cases and hyperplastic nodules. Spearman rank correlation analysis was performed to assess the relationships between t- and p-FAK, as well as t- and p-Src immunoreactivity. A two-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

t-FAK positivity was noted in 43 (39.81%) out of 108 cases with thyroid lesions. The pattern of t-FAK distribution was both cytoplasmic and membraneous in all positive cases examined. Normal thyroid tissue was found negative for t-FAK. In Fig. 1, representative t-FAK immunostaining for hyperplastic nodules (Fig. 1a) and papillary carcinoma (Fig. 1b) are depicted. t-FAK positivity was not significantly associated with patients' age and gender (Table 2, $p>0.05$). t-FAK positivity provided a statistically significant discrimination between malignant and benign thyroid lesions (Table 2, $p=0.00001$). t-FAK immunoreactivity, expressed by the percentage of t-FAK positively stained follicular cells, was significantly increased in malignant (mean % t-FAK positive staining: 29.27±32.50%) compared to benign (mean % t-FAK positive staining: 6.98±18.53%) thyroid lesions (Fig. 2, $p=0.00001$). The box-whisker plot of t-FAK immunoreactivity in benign and malignant thyroid lesions is depicted in Fig. 2a. Thyroid lesions with high proliferative capacity of follicular cells, reflected by Ki-67 labelling index, showed significantly increased incidence of t-FAK positivity (Table 2, $p=0.0003$).

Stratifying for the most frequently apparent types of benign and malignant thyroid lesions, t-FAK positivity provided a statistically significant discrimination between cases with papillary carcinoma and those with hyperplastic nodules (Table 2, $p=0.00005$). FAK immunoreactivity, expressed by the percentage of t-FAK positively stained follicular cells, was significantly increased in cases with papillary carcinoma (mean % t-FAK positive staining: 28.36±33.56%) compared to those with hyperplastic nodules (mean % t-FAK positive staining: 5.13±16.24%) ($p=0.00005$). The box-whisker plot of t-FAK immunoreactivity in papillary carcinoma and hyperplastic nodule cases is depicted in Fig. 2b. Twenty-five (56%) out of 45 papillary, 6 (75%) out of 8 medullary, 3 (60%) out of 5 follicular and one (50%) out of 2 anaplastic carcinoma cases stained positively for t-FAK (Table 3). In contrast, 34 (87%) out of 39 hyperplastic nodules and 6 (67%) out of 9 Hashomoto thyroiditis cases were t-FAK negative (Table 3).

We also assessed whether t-FAK positivity bears a pronounced diagnostic effect in the subgroup of patients with malignant thyroid lesions (Table 4). We stratified by pT stage (small vs large tumor size, pT1 vs pT2-4), lymph nodal status (absence vs presence of lymph node metastases, pN0 vs pN1), capsular, lymphatic and vascular invasion (absence vs presence of invasion). In cross-tables, the incidence of t-FAK positivity was significantly increased in patients presenting large tumor size (Table 4, $p=0.0455$), presence of capsular (Table 4, $p=0.0102$) and lymphatic (Table 4, $p=0.0173$) invasion, while a trend of correlation with vascular invasion (Table 4, $p=0.0801$) was also noted. t-FAK-positive cases more frequently presented high follicular cells' proliferative capacity compared to negative ones without reaching statistical significance (Table 4, $p=0.1062$). In contrast to malignant thyroid cases, t-FAK positivity was significantly associated with follicular cells' proliferative capacity ($p=0.0442$) in benign ones.

t-Src positivity was noted in 59 (54.63%) out of 108 thyroid lesions cases. The pattern of t-Src distribution was both cytoplasmic and membraneous in all positive cases examined. Normal thyroid tissue was found negative for t-Src. In Fig. 1, representative t-Src immunostaining for hyperplastic nodules (Fig. 1c) and papillary carcinoma (Fig. 1d) are depicted. t-Src positivity was not significantly associated with patients' age and gender, and Ki-67 protein statement (Table 2, $p>0.05$). The incidence of t-Src positivity was increased in malignant thyroid lesions compared to benign ones; however, the difference was not statistically significant (Table 2, $p>0.05$). t-Src immunoreactivity, expressed by the percentage of t-Src positively stained follicular cells, was increased in malignant (mean % t-Src positive staining: 20.80±22.61%) compared to benign

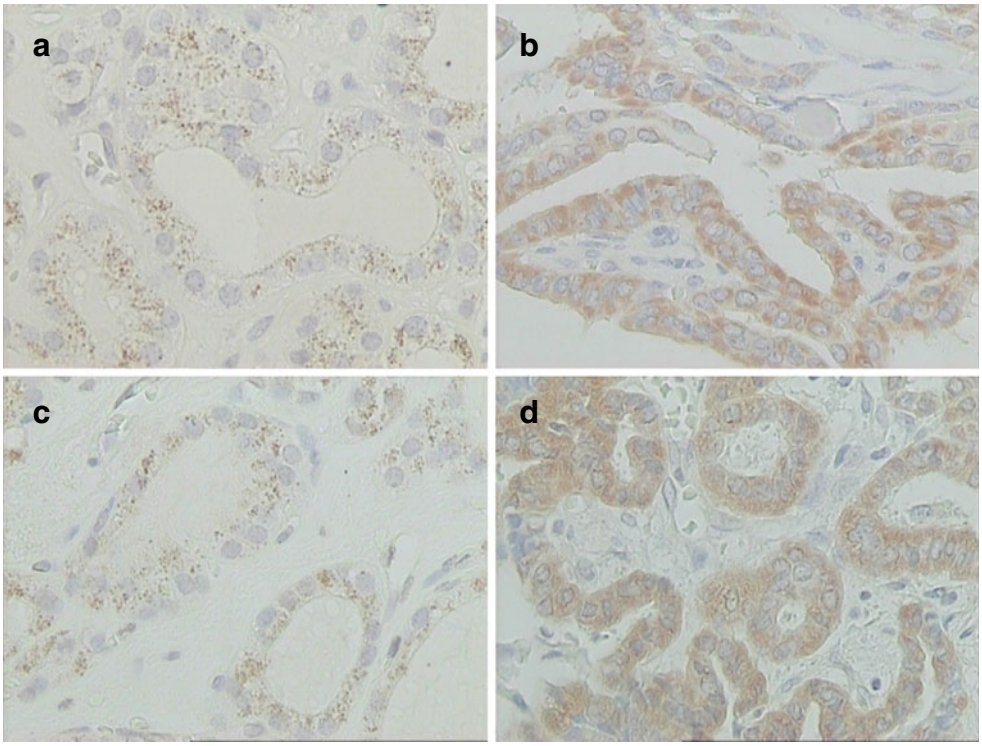

Fig. 1 Representative immunostainings for FAK in cases with **a** Hyperplastic nodules and **b** Papillary carcinoma (original magnification X400) and for Src in cases with **c** Hyperplastic nodules and **d** Papillary carcinoma (original magnification X400)

(mean % t-Src positive staining: 15.72±19.32%) thyroid lesions without reaching statistical significance (Fig. 3a, $p>0.05$). The box-whisker plot of t-Src immunoreactivity in benign and malignant thyroid tumors is depicted in Fig. 3a.

The incidence of t-Src positivity was borderline increased in cases of papillary carcinoma compared to those of hyperplastic nodules (Table 2, $p=0.0993$). t-Src immunoreactivity, expressed by the percentage of Src positively stained follicular cells was significantly increased in papillary carcinoma (mean % t-Src positive staining: 25.84±23.03%) compared to hyperplastic nodules cases (mean % t-Src positive staining: 14.48±17.68%) ($p=0.0167$). The box-whisker plot of t-Src immunoreactivity in papillary carcinoma and hyperplastic nodules cases is

Table 2 Associations of t-FAK and t-Src expression with patients' age and gender, type of histopathology (Benign vs Malignant thyroid lesions) and Ki-67 protein statement in 108 cases with thyroid lesions

Clinicopathological Characteristics	t-FAK expression			t-Src expression		
	Negative (%)	Positive (%)	*p*-value	Negative (%)	Positive (%)	*p*-value
***N*=108**	65 (60.19)	43 (39.81)		49 (45.37)	59 (54.63)	
Age (mean±SD;ys)	51.46±14.26	53.70±14.59	0.3926	50.77±13.12	54.18±14.65	0.3183
Gender						
Female	50 (46.30)	38 (35.19)	0.1338	38 (35.19)	50 (46.30)	0.3379
Male	15 (13.89)	5 (4.63)		11 (10.19)	9 (8.33)	
Histopathology						
Benign	40 (37.04)	8 (7.41)	**0.00001**	23 (21.30)	25 (13.15)	0.6345
Malignant	25 (23.15)	35 (32.41)		26 (24.07)	34 (31.48)	
Ki-67 protein statement						
< mean value	52 (48.15)	20 (18.52)	**0.0003**	31 (28.70)	41 (37.96)	0.4944
≥ mean value	13 (12.04)	23 (21.30)		18 (16.67)	18 (16.67)	
Histopathology (*n*=84)			**0.00005**			0.0993
Hyperplastic nodules	34 (40.48)	5 (5.95)		19 (22.62)	20 (23.81)	
Papillary carcinoma	20 (23.81)	25 (29.76)		14 (16.67)	31 (36.90)	

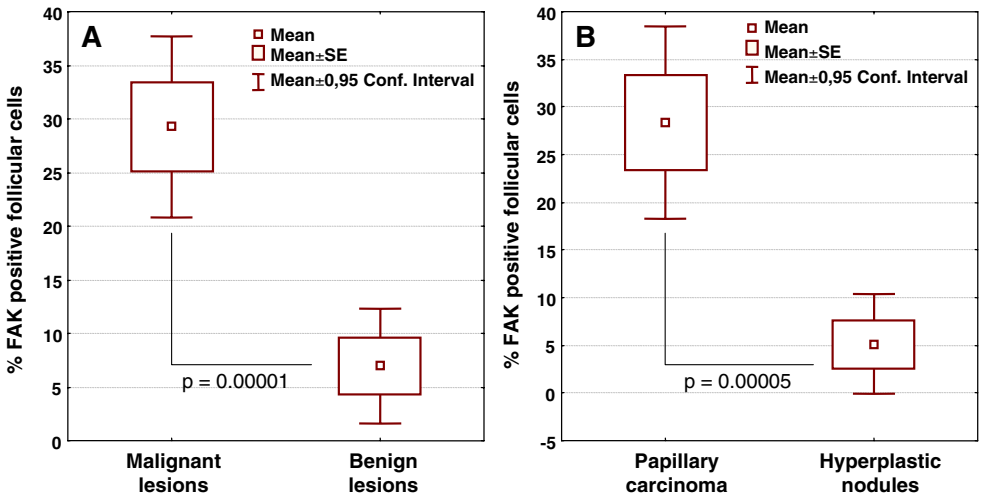

Fig. 2 Box-whisker plots of FAK immunoreactivity: **a** Benign vs Malignant thyroid lesions and **b** Hyperplastic nodules vs Papillary carcinomas

depicted in Fig. 3b. Thirty-one (69%) out of 45 papillary carcinoma cases were t-Src positive, whereas only 20 (51%) out of 39 hyperplastic nodules stained positively for t-Src (Table 3). In contrast to papillary carcinomas, 7 (88%) out of 8 medullary, 3 (60%) out of 5 follicular and 2 (100%) out of 2 anaplastic carcinoma cases were negative for t-Src (Table 3). Hashimoto thyroiditis cases showed a similar incidence of t-Src positivity to hyperplastic nodules, as 5 (56%) out of 9 Hashimoto thyroiditis cases stained positively for t-Src (Table 3).

We also assessed whether t-Src positivity bears a pronounced diagnostic effect in the subgroup of patients with malignant thyroid lesions (Table 4). In cross-tables, the incidence of t-Src positivity was significantly reduced in patients presenting large tumor size (Table 4, p=0.0169). t-Src positivity was not significantly associated with lymph nodal status, Ki-67 protein statement and the presence of capsular, lymphatic and vascular invasion (Table 4, p>0.5).

Spearman rank correlation analysis was further performed to assess the relationships between total and phosphorylated forms of FAK and Src percentage immunoreactivity. Specimens presenting negative immunoreactivity for t-FAK and t-Src also proved negative for the phosphorylated form of the enzymes. In t-FAK and t-Src positive cases, the immunoreactivity for their phosphorylated forms was reduced in a systematic way (Fig. 4a and b, respectively). In particular, specimens presenting reduced t-FAK and t-Src percentage expression values showed even lower or negative immunoreactivity for the corresponding phosphorylated forms of the enzymes. Accordingly, specimens presenting increased t-FAK and t-Src percentage expression values showed lower immunoreactivity for p-FAK and p-Src, respectively (Fig. 4a and b, respectively). p-FAK staining showed an equal discrimination between malignant and benign thyroid lesions (mean p-FAK % staining: 16.33±24.99% vs 1.25±4.55%, p=0.0001), as well as between papillary carcinoma and hyperplastic nodules cases (mean p-FAK % staining: 18.67±26.01% vs 1.28±4.83%, p=0.00009) compared to that provided by t-FAK staining (Fig. 5a). p-Src staining showed an even better discrimination between malignant and benign thyroid lesions (mean p-Src % staining: 15.42±19.94% vs 3.33±7.81%, p=0.0005), as well as between papillary carcinoma and hyperplastic nodules cases (mean p-Src % staining: 19.00±20.77% vs 3.08±7.66%, p=0.00004) compared to that provided by total Src staining (Fig. 5b).

Discussion

The role of both tyrosine kinases, FAK and Src in human malignancy has been well established and different current

Table 3 t-FAK and t-Src expression in distinct groups of benign and malignant thyroid lesions

Thyroid lesions	N	t-FAK expression		t-Src expression	
		Negative (%)	Positive (%)	Negative (%)	Positive (%)
Papillary carcinoma	45	20 (44)	25 (56)	14 (31)	31 (69)
Medullary carcinoma	8	2 (25)	6 (75)	7 (88)	1 (12)
Follicular carcinoma	5	2 (40)	3 (60)	3 (60)	2 (40)
Anaplastic carcinoma	2	1 (50)	1 (50)	2 (100)	0 (0)
Hyperplastic nodule	39	34 (87)	5 (13)	19 (49)	20 (51)
Hashimoto thyroiditis	9	6 (67)	3 (33)	4 (44)	5 (56)

Table 4 Associations of t-FAK and t-Src expression with clinicopathological characteristics in patients with malignant thyroid lesions

Clinicopathological Characteristics	t-FAK expression			t-Src expression		
	Negative (%)	Positive (%)	*p*-value	Negative (%)	Positive (%)	*p*-value
***N*=60**	25 (41.67)	35 (58.33)		26 (43.33)	34 (56.67)	
Tumor size (T)			**0.0455**			**0.0169**
T1	21 (35.00)	21 (35.00)		14 (23.33)	28 (46.67)	
T2-4	4 (6.67)	14 (23.33)		12 (20.00)	6 (10.00)	
Lymph node metastasis (N)			0.1179			0.4328
N0	24 (40.00)	29 (48.33)		22 (36.67)	31 (51.67)	
N1	1 (1.67)	6 (10.00)		4 (6.67)	3 (5.00)	
Capsular invasion			**0.0102**			0.7206
No	25 (41.67)	27 (45.00)		23 (38.33)	29 (48.33)	
Yes	0 (0)	8 (13.33)		3 (5.00)	5 (8.33)	
Lymphatic invasion			**0.0173**			0.4017
No	25 (41.67)	28 (46.67)		24 (40.00)	29 (48.33)	
Yes	0 (0)	7 (11.67)		2 (3.33)	5 (8.33)	
Vascular invasion						
No	25 (41.67)	31 (51.67)	0.0801	24 (40.00)	32 (53.33)	0.7806
Yes	0 (0)	4 (8.51)		2 (3.33)	2 (3.33)	
Ki-67 protein statement						
< mean value	16 (26.67)	15 (25.50)	0.1062	12 (20.00)	19 (31.67)	0.4549
≥ mean value	9 (15.00)	20 (33.33)		14 (23.33)	15 (25.00)	

research projects are directed to evaluate their diagnostic and prognostic utility in human neoplasia [20, 33]. Moreover, a considerable number of small-molecule tyrosine kinase inhibitors which target FAK and Src enzymes have currently been introduced in clinical development to evaluate their clinical efficiency in anticancer therapy [34–36]. However, the available data concerning the clinical significance of FAK and Src expression in thyroid tissue lesions remains limited so far. Under this consideration, the current study revealed that both proteins can be expressed in both benign and malignant thyroid tissue lesions, having an incidence for t-FAK positivity equal to 39.81% of the examined cases and an even higher incidence for t-Src positivity equal to 54.63% of the examined cases. t-FAK and t-Src positivity was ascribed to a significant extent to the phosphorylated forms of the enzymes, supporting evidence that both of them are present in their activated forms within thyroid lesions. The higher percentage expression value of p-FAK and p-Src in malignant compared to benign thyroid lesions reflects that both enzymes are in active form, which may reinforce their potential use as therapeutic targets in thyroid neoplasia.

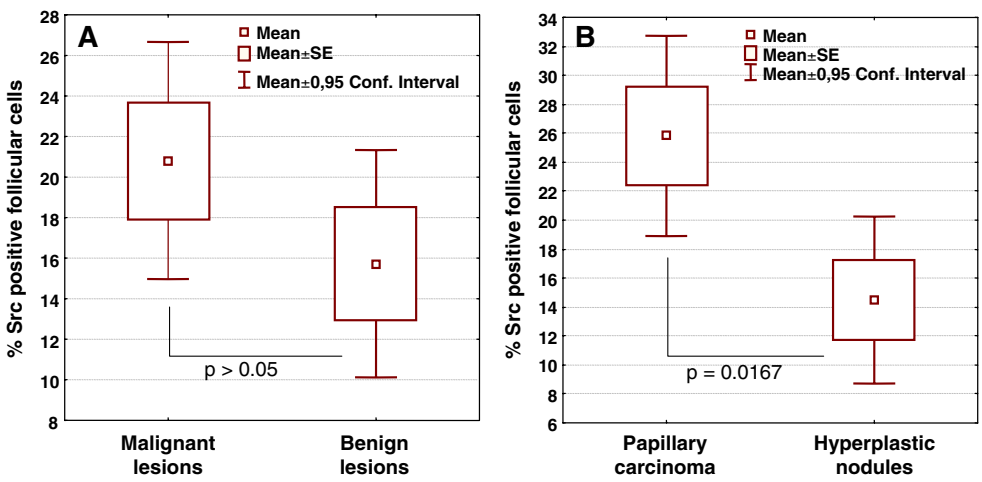

Fig. 3 Box-whisker plots of Src immunoreactivity: **a** Benign vs Malignant thyroid lesions and **b** Hyperplastic nodules vs Papillary carcinomas

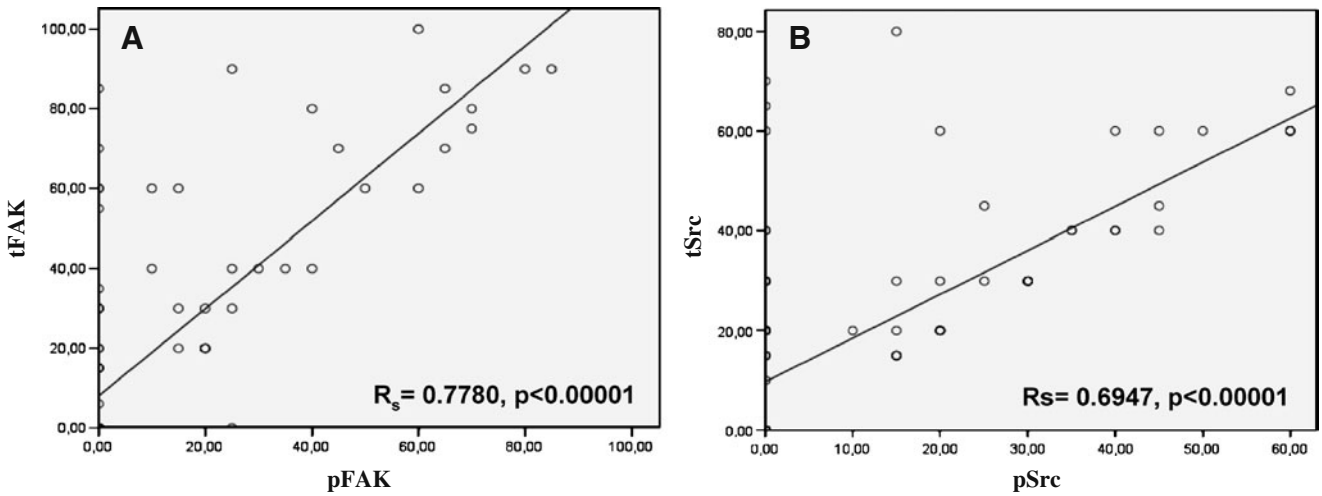

Fig. 4 Spearman rank correlations between total and phosphorylated **a** FAK and **b** Src percentage expression values

t-FAK expression in contrast to t-Src was found to provide a distinct discrimination between malignant and benign thyroid lesions. In fact, 40 (83.33%) out of 48 benign thyroid cases examined were found t-FAK negative, whereas 35 (58.33%) out of 60 malignant thyroid carcinomas were t-FAK positive. Similarly, t-Src expression was increased in malignant compared to benign thyroid lesions without though reaching statistical significance. In agreement with the present study, Kim et al. documented that t-FAK immunoexpression was significantly increased in malignant compared to benign thyroid lesions [27]. In particular, all hyperplastic nodules (6 out of 6 cases) and 8 (53.33%) out of 15 folicullar adenomas were found t-FAK negative, whereas all malignant cases (17 papillary, 9 folicullar, 8 medullary and 2 anaplastic carcinomas) were t-FAK positive; however, it should be noted that this study was performed on a lower number of cases, including in total 21 benign and 36 malignant thyroid lesions [27]. Owens et al. also assessed by western blot the levels of

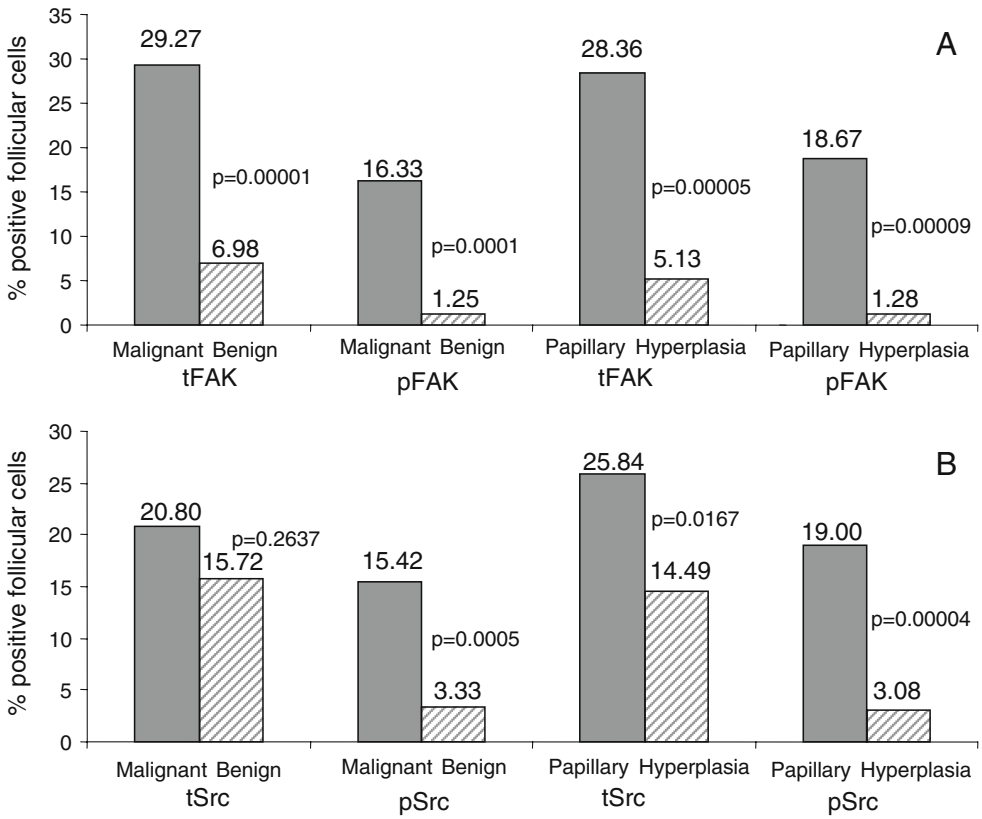

Fig. 5 Histograms of total and phosphorylated **a** FAK and **b** Src % positive follicular cells immunostaining in benign vs malignant, as well as papillary carcinoma vs hyperplastic nodules thyroid lesions

t-FAK in 30 patients with thyroid lesions [26]. In this study, t-FAK expression was directly associated with the aggressive phenotype of thyroid carcinomas. In fact, the highest levels of t-FAK were seen in follicular carcinomas and in thyroid tumors associated with distant metastatic foci. In contrast, malignant thyroid tissues with reduced invasive potential, such as papillary, follicular adenomas and other benign thyroid lesions showed minimal t-FAK expression [26]. Interestingly, we found that both t-FAK and t-Src immunoreactivity was significantly increased in papillary carcinoma cases compared to hyperplastic nodules. The data above supports evidence that both proteins could be considered of clinical utility in thyroid lesions. Further research effort should be conducted on larger series to evaluate FAK and Src expression in distinct malignant thyroid lesions, such as papillary, follicular, medullary and anaplastic carcinoma. Moreover, as both FAK and Src appear to be elevated in malignant thyroid lesions and especially in papillary carcinoma, their expression could be considered clinically important for future therapeutic approaches.

The incidence of t-FAK positivity appeared to increase significantly in thyroid lesions with enhanced follicular cells' proliferative capacity. Our finding is in line with previous evidence in which FAK-overexpressing tumor cells exhibited enhanced proliferating capacity compared to non-overexpressing ones in several types of cancer, such as esophageal, gastric and breast cancer [30, 37, 38]. There is also substantial evidence which supports that FAK may contribute to uninhibited proliferation of cancer cells mainly through the Extracellular-regulated kinase (Erk) signaling pathway [13, 35]. Indeed, in human glioblastoma cells, FAK overexpression, in vivo, promoted Erk activity and increased the transcription of the Kuppel-like factor 8 (KLF8), which directly activated cyclin-D1 transcription and thus promoted cell proliferation [11]. Moreover, the FAK dominant negative, FAK-related non-kinase (FRNK), expression suppressed the growth of human tumor cells in nude mice [39].

In the current study, the incidence of t-FAK positivity was significantly increased in patients with large tumor size and presence of capsular and lymphatic invasion. In this context, recent in vitro evidence has suggested that FAK may play a crucial role in promoting cell invasion through the activation of distinct signaling pathways induced by epidermal growth factor (EGF) with protein MMP-9 transcription and secretion in follicular thyroid carcinoma cells [28]. Accordingly, in situ evidence also revealed significant association of FAK immunoreactivity with tumor size in several types of neoplasia [40–42]. More to the point, FAK overexpression assessed by immunohistochemistry, was significantly correlated with large tumor size in liver and pancreatic cancer, as well as in intrahepatic cholangiocarcinoma [40–42]. FAK expression assessed by both Western blot and real-time PCR, was also significantly associated with tumor size in non-small cell lung cancer patients [43]. It was also reported that astrocytoma cells expressing FAK formed larger tumors in nude mice than in tumor cells derived from the parental cell lines [44], while expression of a hyperactive mutant of FAK in a breast cancer cell line resulted in elevated tumor size in nude mice [45]. There is also evidence that enhanced FAK immunoreactivity was associated with advanced disease stage and presence of lymph node metastasis in esophageal, breast, lung, ovarian and colon carcinoma [37, 38, 42, 46, 47]. On the other hand, the current study further revealed that the incidence of t-Src positivity was significantly reduced in patients presenting large tumor size, while no significant associations with the other clinicopathological parameters were noted. In this context, it was shown that Src expression was inversely correlated with Ki-67 labeling index, TNM stage, tumor size, and histopathological grade in patients with hepatocellular and breast carcinoma, suggesting that Src may play an important role in the early progression of hepatocellular and breast carcinoma [48, 49].

Conclusions

The current study revealed for the first time that FAK immunoreactivity may exhibit clinical utility, providing a distinct discrimination between benign and malignant thyroid lesions, being also associated with the proliferative capacity of follicular cells and with important clinicopathological parameters for patients' management. Src immunoreactivity proved of lower clinical value. Further research effort should be conducted on larger series to evaluate whether FAK and Src immunoreactivity can distinguish the different types of malignant thyroid tumors. It should also be taken into consideration that although immunohistochemistry offer broader applicability, it is affected by the sensitivity of the antibodies used, the type of tissue examines (frozen vs formalin fixed) and the various scoring and interpretative criteria used to evaluate the cases. Thus, further studies are warranted to confirm the diagnostic and prognostic utility of FAK and Src by more sensitive techniques, such as real-time PCR and Western blotting. Further research effort focused on the clinical significance of the activated-phosphorylated forms of FAK and Src is required to delineate whether they could be considered of diagnostic and prognostic utility and may exhibit targeted therapeutic potential in thyroid neoplasia.

Conflict of interest statement We declare that we have no conflict of interest.

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