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ORIGINAL ARTICLE

FAK, Src and p-Paxillin expression is decreased in liver metastasis of colorectal carcinoma patients

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Summary

Purpose: *The FAK/Src/Paxillin (PXN) axis has been implicated in malignant transformation, tumor growth, progression and metastasis. The present study aimed to assess FAK/Src/PXN protein expression in both primary and liver metastatic sites of colorectal adenocarcinoma (CRC).*

Methods: *FAK, Src and p-PXN expression was assessed immunohistochemically on 32 primary CRCs and their corresponding liver metastases, being also analyzed in relation with clinicopathological characteristics and patient survival.*

Results: *FAK, Src and p-PXN expression was significantly decreased in liver metastasis compared to matched paired primary CRCs ($p<0.01$). Increased FAK expression in primary CRCs was significantly associated with poor histological grade and advanced disease stage ($p=0.0330$ and $p=0.0204$, respectively). Increased Src expression in primary colorectal tumors was significantly associated with the presence of lymph node metastasis ($p=0.0325$), while elevated p-PXN expression with poor histological grade ($p=0.0284$).*

Conclusions: *FAK, Src and p-PXN appear to play a role in the pathophysiological aspects of CRC. The lower expression of these proteins in liver metastasis compared to the primary CRC could significantly impact the choice of a novel therapeutic agent according to the disease stage.*

Key words: *clinicopathological parameters, colorectal carcinoma, FAK, liver metastasis, Paxillin, Src*

Introduction

Focal Adhesion Kinase (FAK), alternatively known as Protein Tyrosine Kinase 2 (PTK2), and proto-oncogene tyrosine protein kinase Src are ubiquitously expressed non-receptor tyrosine kinases, members of the tyrosine kinase family [1]. FAK is a central regulator of integrin-mediated cell adhesion and migration , while it participates in downstream signaling pathways of other cell receptors, including G-protein coupled receptors (GPCRs), low density lipoprotein (LDL) receptors and growth factor receptors. FAK is activated by phosphorylation at tyrosine residue Y397, which promotes its binding to Src, subsequently leading to FAK phosphorylation at Y576 and Y577, a step which is required for its maximal kinase activity [2]. Reciprocally, Src is also activated through phosphorylation at Y416 [3]. The activated FAK/Src dual kinase complex then initiates multiple phosphorylation cascades, regulating a number of cellular functions such as cell adhesion and migration, angiogenesis, cell cycle, cell proliferation and apoptosis. A prominent substrate of the FAK/

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Src kinase complex is Paxillin (PXN), a cytoskeletal scaffolding protein which interacts with several structural and regulatory proteins important for coordinating changes in the actin cytoskeleton associated with cell motility and cell adhesion. PXN binds to and is phosphorylated at Y118 by the FAK/Src kinase complex [4].

The involvement of FAK/Src complex in cellular pathways that regulate cell growth and motility suggests that they may contribute to the development of cancer and indicates these proteins as putative drug targets for cancer treatment [5]. Many studies using either *in vitro* or animal models have implicated FAK/Src signaling in malignant transformation, tumor growth and progression, as well as metastasis. FAK/Src signaling has also been reported to promote cell survival through inhibition of the cellular tumor antigen p53 and/or phosphatidylinositol 3-kinase-mediated activation of AKT, as well as cell invasion, through activation of Rac and Rho GTPases, and also as matrix metalloproteases [6]. In addition, clinical studies have revealed that FAK and/or Src expression is significantly negatively correlated negatively with clinicopathological parameters and patients' survival in many cancer types [7,8]. Similarly, PXN has been implicated in tumor progression, angiogenesis and metastasis [9-11], either as a FAK/Src downstream target or on its own accord. Moreover, PXN expression has been associated with poor clinicopathological parameters and poor patients' survival in various cancer types [12-14].

Liver metastases is the main cause of death in CRC patients. In this aspect, significant research is aimed at elucidating the molecules participating in metastatic mechanisms and pathways. It is widely accepted that certain steps are required in order for metastases to be widespread [15]. These include epithelial-mesenchymal transition (EMT) and breach of the basic membrane, dissociation of tumor cells from the main tumor, invasion of neighboring tissues, intravasation in blood and lymph vessels, transport and extravasation of tumor cells, establishment in a distant site and formation of micro- and macro-metastases. In these steps, FAK/Src complex and signaling are essential for tumor cell adhesion to the extracellular matrix (ECM) and its remodeling during the invasion and migration of tumor cells [16]. Furthermore, the activation of integrin and its downstream kinases, including FAK and Src protects tumor cells from anoikis [16,17]. High levels of FAK have been shown to stimulate tumor cell proliferation, migration and survival [18]. Also, the metastatic phenotype is promoted by angiogenesis and by hypoxia-inducible factors like HIF1A which mediate EMT by upregulating lysyl oxidase (LOX) and by activating FAK in intratumoral hypoxic conditions [19].

Little data are available regarding the expression of FAK, Src and PXN in metastatic tumors. In this aspect, the present study aimed to assess immunohistochemically the expression of FAK, Src and p-PXN in 32 primary CRCs and their corresponding hepatic metastases, in association with clinicopathological parameters, as well as overall patients' survival. Tumor proliferative capacity was assessed by measuring Ki-67 labelling index in both primary and metastatic sites.

Methods

Patients

Thirty-two patients constituted the group of our study, with samples from primary CRCs and their corresponding liver metastases. Nineteen patients were male (59.4%) and 13 female (40.6%) with a mean age of 65.4 years (range 42-83). Eight tumors (25%) were located in the right colon, 11 (34.4%) in the left and 13 (40.6%) in the rectum. Twenty-five patients (78.1%) were originally staged as stage IV, while 7 (21.9%) presented metachronous metastases (initially stages I-III) within more than 6 months of initial diagnosis. Among initially non-metastatic tumors, 5 (71.4%) were classified as node negative (N0), while 2 (28.6%) were classified as node positive (1 N1, 1 N2). Among initially metastatic tumors, 4 (16%) were classified as node negative, while 21 (84%) as node positive. In total, 9 primary tumors (28.1%) were classified as node negative (N0), while 23 (71.9%) as node positive (9 N1, 14 N2). Primary tumor differentiation was classified as moderate in 23 patients (71.9%) and as low in 9 patients (28.1%).

Immunohistochemistry

Immunostaining for FAK, Src and p-PXN was performed on formalin-fixed, paraffin-embedded tissue sections using a mouse anti-human FAK IgG_1 antibody, raised against the COOH-terminal of total FAK protein (sc-1688, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse anti-human total c-Src IgG_{2a} antibody (sc-5266, Santa Cruz Biotechnology) and a rabbit anti-human polyclonal p-PXN IgG antibody (Tyr118, sc-101774 Santa Cruz Biotechnology), respectively. Briefly, 4μm thick tissue sections were deparaffinized, rehydrated, immersed in 3% H_2O_2 for 30 min, microwaved at 750W in 0.01M citrate buffer (pH 6.0) for 15 min and left to cool down in TBS. Sections were incubated with anti-FAK, anti-Src and anti-p-PXN antibodies for 1 hr at room temperature (37°C), at a dilution 1:200. After washing three times with PBS, sections were then incubated at room temperature with biotinylated linking reagent (Biocare Medical Walnut Greek, CA, USA) for 10 min, followed by incubation with peroxidase-conjugated streptavidin label (Biocare Medical) for 10 min. The resultant immune peroxidase activity was developed using a DAB substrate kit (Vector Laboratories, California, USA) for 7 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, endometrial and thyroid carcinomas tissue sections with known increased FAK, Src and p-PXN immunoreactivity were used [20-22]. A mouse anti-human Ki-67 antigen IgG1k antibody (clone MIB-1, Dakopatts, Glostrup, Denmark) was also used to assess tumor proliferative capacity as it has been previously described by our group [21].

Evaluation of immunohistochemistry

Immunohistochemical evaluation was performed by counting at least 1000 tumor cells in each case by two independent observers (S.T. and E.P.) blinded to the clinical data, with complete observers' agreement. Specimens were considered "positive" for FAK, Src and p-PXN when the percentage of positively stained tumor cells in the section was more than 5%. The immunoreactivity of the tumor cells for FAK, Src and p-PXN was scored according to the percentage of FAK, Src and p-PXN positive tumor cells as 0: negative staining; 0-4% of cells positive; 1: 5-24% of cells positive; 2: 25-49% of cells positive; 3: 50-100% of cells positive, and its intensity as 0: negative staining; 1: mild staining; 2: intermediate staining; 3: intense staining expression in colon carcinoma cells [23-25]. FAK, Src and p-PXN expressions were classified as low, if the total score was 0 or 2, and high, if the total score was ≥3 . In this way, it is ensured that each group has a sufficient and more homogeneous number of cases in order to be comparable with the other groups [23-25].

Statistics

Chi-square test was used to assess the associations of FAK, Src and p-PXN expression with clinicopathological variables and Ki-67 labelling index. Wilcoxon matched paired test was used to compare the expression of FAK, Src and p-PXN between the primary CRCs and the matched paired secondary tumors of liver metastasis. 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 markers and overall patient survival. Cox regression analysis was conducted at both univariate and multivariate levels. A p value less than 0.05 was considered as the limit of statistical significance. SPSS for Windows Software was used for all analyses (SPSS Inc., 2003, Chicago, USA).

Results

All primary CRCs were found positive for FAK and Src (100%), while p-PXN positivity was noted in 28 (87.5%) out of 32 primary CRCs. Twelve (37.5%), 16 (50.0%) and 15 (46.9%) out of 32 primary CRCs showed high FAK, Src and p-PXN expression, respectively. The pattern of FAK, Src and p-PXN distribution in colorectal carcinomas was predominantly cytoplasmic and occasionally membranous. Representative FAK, Src and p-PXN immunostainings in CRCs are depicted in Figure 1.

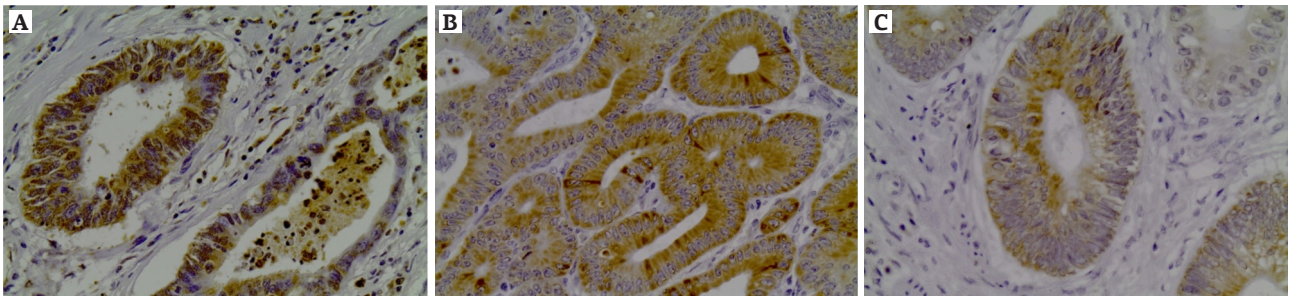

Figure 1. Representative immunostainings for FAK **(A)**, Src **(B)** and p-PXN **(C)** protein expression in tumor cells of primary colon adenocarcinoma. Streptavidin-biotin-peroxidase, DAB chromogen, Harris hematoxylin counterstain (original magnification x400).

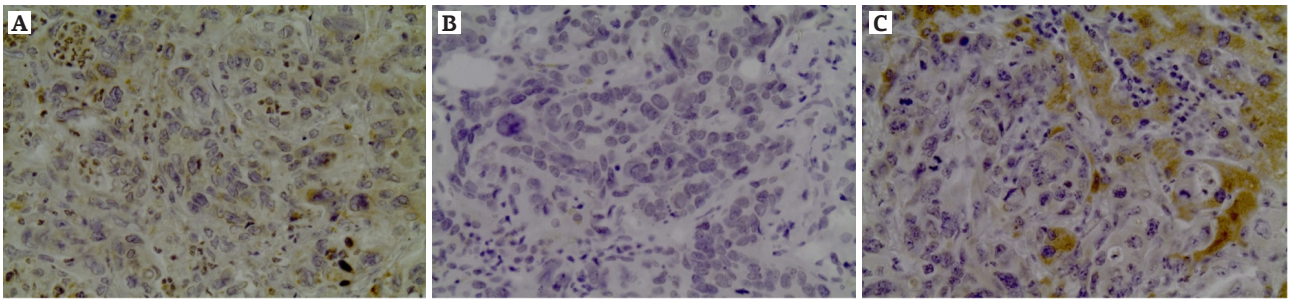

Figure 2. Representative immunostainings for FAK **(A)**, Src **(B)** and p-PXN protein **(C)** expression in tumor cells of secondary liver metastases. Streptavidin-biotin-peroxidase, DAB chromogen, Harris hematoxylin counterstain (original magnification x400).

FAK positivity was noted in 29 (90.6%) out of 32 secondary tumors of liver metastasis. Twenty-two (68.8%) out of 32 secondary tumors of liver metastasis were found positive for Src and 21 (65.6%) secondary tumors were found positive for p-PXN. Nine (28.1%), 10 (31.2%) and 4 (12.3%) out of 32 secondary tumors of liver metastasis showed high FAK, Src and p-PXN expression, respectively. The pattern of FAK, Src and p-PXN distribution in liver metastases was predominantly cytoplasmic and occasionally membranous. Representative FAK, Src and p-PXN immunostainings in liver metastases are depicted in Figure 2.

In cross-tables, increased FAK expression in primary tumors was significantly associated with high histological grade (Table 1, p=0.0330) and advanced disease stage (Table 1, p=0.0204). Increased FAK expression in primary tumors was also more frequently observed in CRC patients presenting organ metastasis at a no significant level though (Table 1, p=0.0976). Increased Src expression in primary tumors was significantly associated with the presence of lymph node metastasis (Table 2, p=0.0325). Increased p-PXN expression in primary tumors was also significantly more frequently observed in CRCs patients with high histological grade (Table 3, p=0.0284).

Kaplan-Meier survival curves indicated that CRC patients with high FAK expression in their primary tumor presented shorter survival compared to those with low FAK expression (Figure 3, log-rank test, p=0.0121), while Src and p-PXN expression was not associated with patient survival (Figure 3, log-rank test, p=0.6315 and p=0.7172, respectively). In multivariate analysis, FAK expression was identified as an independent prognostic factor of patient survival (Table 4, Cox regression analysis, p=0.0022). Notably, FAK, Src and p-PXN expressions were significantly decreased in liver metastasis compared to the matched primary colorectal tumors (Figure 4, p<0.01). This rather interesting finding is analysed in the following sections. No difference was observed as far as Ki-67 expression is concerned between primary CRCs and liver metastasis (p=0.894).

Table 1. Correlation of FAK expression with clinicopathological characteristics and Ki-67 labeling index

Clinicopathological characteristics	*FAK expression*		
	Low (%)	*High (%)*	*p value*
n=32	20 (62.5)	12 (37.5)	
Age, years (median 65.5)			1.000
< 65.5	10 (31.2)	6 (18.7)	
≥ 65.5	10 (31.2)	6 (18.7)	
Gender			0.5153
Male	11 (34.4)	8 (25.0)	
Female	9 (28.1)	4 (12.5)	
Histological grade			0.0330
I-II	17 (58.1)	6 (18.7)	
III	3 (9.4)	6 (18.7)	
Tumor size (T)			
T1-3	17 (58.1)	9 (28.1)	0.4829
T4	3 (9.4)	3 (9.4)	
Lymph node metastasis (N)			0.5809
N0-1	12 (37.5)	6 (18.7)	
N2	8 (25.0)	6 (18.7)	
Organ metastasis (M)			0.0976
No	4 (12.5)	0 (0.0)	
Yes	16 (50.0)	12 (37.5)	
TNM stage			0.0204
I-III	7 (21.9)	0 (0.0)	
IV	13 (40.6)	12 (37.5)	
Ki-67 protein			0.3143
< median value	8 (25.0)	7 (21.9)	
≥ median value	12 (37.5)	5 (15.6)	

Table 2. Correlation of Src expression with clinicopathological characteristics and Ki-67 labeling index

Clinicopathological characteristics	*Src expression*		
	Low (%)	*High (%)*	*p value*
n=32	16 (50)	16 (50)	
Age, years (median 65.5)			1.000
< 65.5	8 (25.0)	8 (25.0)	
≥ 65.5	8 (25.0)	8 (25.0)	
Gender			0.7189
Male	9 (28.1)	10 (31.2)	
Female	7 (21.9)	6 (18.7)	
Histological grade			0.2381
I-II	13 (40.6)	10 (31.2)	
III	3 (9.4)	6 (18.7)	
Tumor size (T)			0.3650
T1-3	14 (43.7)	12 (37.5)	
T4	2 (6.3)	4 (12.5)	
Lymph node metastasis (N)			0.0325
N0-1	12 (37.5)	6 (18.7)	
N2	4 (12.5)	10 (31.2)	
Organ metastasis (M)			0.2850
No	1 (3.1)	3 (9.4)	
Yes	15 (46.9)	13 (40.6)	
TNM stage			0.6689
I-III	3 (9.4)	4 (12.5)	
IV	13 (40.6)	12 (37.5)	
Ki-67 protein			0.7231
< median value	8 (25.0)	7 (21.9)	
≥ median value	8 (25.0)	9 (28.1)	

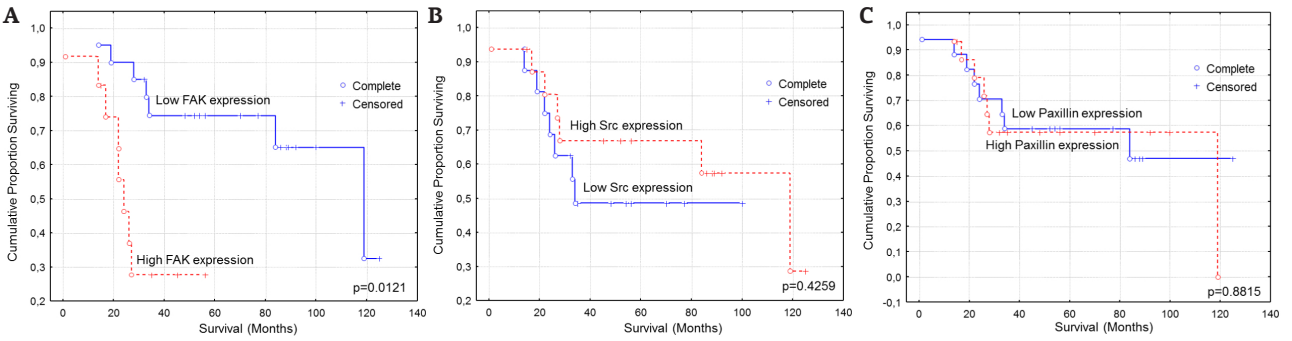

Figure 3. Kaplan-Meier survival analysis stratified according to concomitant FAK **(A)**, Src **(B)** and p-PXN **(C)** protein expression in patients with primary colon adenocarcinoma and overall patient survival.

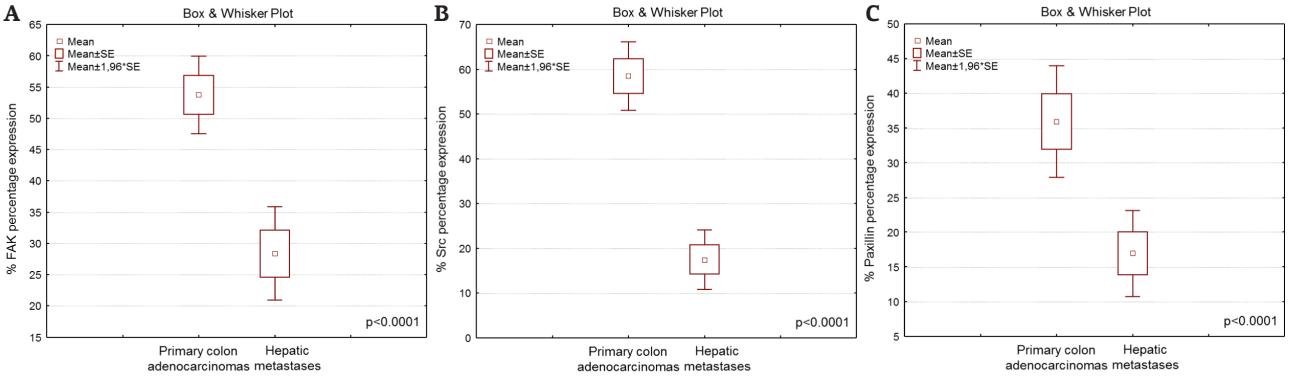

Figure 4. Mann-Whitney graphics regarding FAK **(A)**, Src **(B)** and p-PXN **(C)** expression in liver metastases in comparison to matched paired primary colorectal tumors.

Table 3. Correlation of p-Paxillin expression with clinicopathological characteristics and Ki-67 labeling index

Clinicopathological characteristics	*p-Paxillin expression*		
	Low (%)	*High (%)*	*p value*
n=32	17 (53.1)	15 (46.9)	
Age, years (median 65.5)			0.7231
< 65.5	8 (25.0)	8 (25.0)	
≥ 65.5	9 (28.1)	7 (21.9)	
Gender			0.1691
Male	12 (37.5)	7 (21.9)	
Female	5 (15.6)	8 (25.0)	
Histological grade			0.0284
I-II	15 (46.9)	8 (25.0)	
III	2 (6.2)	7 (21.9)	
Tumor size (T)			0.8648
T1-3	14 (43.7)	12 (37.5)	
T4	3 (9.4)	3 (9.4)	
Lymph node metastasis (N)			0.7547
N0-1	10 (31.2)	8 (25.0)	
N2	7 (21.9)	7 (21.9)	
Organ metastasis (M)			0.3486
No	3 (9.4)	1 (3.1)	
Yes	14 (43.7)	14 (43.7)	
TNM stage			0.5379
I-III	3 (9.4)	4 (12.5)	
IV	14 (43.7)	11 (34.4)	
Ki-67 protein			0.9823
< median value	8 (25.0)	7 (21.9)	
≥ median value	9 (28.1)	8 (25.0)	

Table 4. Cox multivariate survival analysis in relation with FAK protein expression

Clinicopathological variables	*Overall disease free survival*	
	HR (95% CI)	*p value*
Age, years		
(< 65.5 / ≥ 65.5)	1.206 (0.095-3.948)	0.7621
Gender		
(Male /Female)	0.932 (0.066-4.103)	0.9225
Histopathological grade		
(I+II / III)	0.380 (0.028-2.939)	0.3777
pT		
(T1-3 / T4)	2.179 (1.021-4.893)	0.3660
pN		
(N0-1 / N2)	5.648 (2.647-8.938)	0.0493
pM		
(No / Yes)	2.001 (0.783-5.839)	0.5680
Ki-67 protein		
(< median value / ≥ median value)	3.652 (1.932-5.722)	0.1001
FAK expression		
(Low / High)	16.816 (12.473-19.438)	0.0022

Discussion

The importance of FAK, Src and PXN in human malignancy has been previously established [7-11]. Moreover, previous studies in sarcomas, breast, colon and other tumors have found significantly elevated FAK levels in invasive and metastatic lesions, as well as in preinvasive lesions, while non-invasive and normal tissues showed weak or absent FAK expression [26,27]. Other previous studies have documented a significant increase in FAK and Src protein expression levels in well-differentiated compared to poorly differentiated tumors [25]. FAK has been associated with advanced tumor grade in astrocytomas, oral SCC, laryngeal SCC, lung, esophageal, gastric, hepatocellular, pancreatic, endometrial, ovarian, breast cancer and soft tissue tumors. FAK has been associated with advanced TNM stage in oral SCC, non-small cell lung, esophageal, colon, hepatocellular, pancreatic, ovarian, breast cancer and soft tissue tumors [7,20]. On the contrary, concomitant FAK/Src expression was associated with high tumor grade of differentiation in mobile tongue squamous cell carcinoma [28]. Elevated Src has been associated with advanced tumor grade in lung, colorectal, hepatocellular (pSrc), endometrioid subtype of endometrial (pSrc) and breast (tSrc and pSrc) cancer [8,21]. Elevated Src has been also associated with advanced stage in tongue cancer (pSrc), malignant pleural mesothelioma (pSrc) and pancreatic ductal adenocarcinoma (tSrc) [8,20]. On the contrary in other studies, elevated Src has been correlated with low tumor grade in hepatocellular (pSrc) and breast cancer (tSrc) [8]. Elevated Src has been also associated with early tumor stage in pancreatic adenocarcinoma (tSrc) and breast cancer (tSrc) [8]. Elevated Paxillin has been associated with advanced tumor grade in laryngeal SCC [29], colorectal cancer [12,30], glioblastoma [31], gallbladder squamous cell/adenosquamous and adenocarcinomas [32]. Elevated PXN has been also associated with advanced TNM stage in laryngeal SCC [29], salivary adenoid cystic carcinoma [33], gastric cancer [13], colorectal cancer [12,30], gallbladder squamous cell/adenosquamous and adenocarcinomas [32].

Most studies in the literature are designed to assess protein levels at the primary tumor site in a specific disease stage. This however doesn't always fully reflect the effects of the same protein, since in metastases the expression can be entirely different. As mentioned above, there is only a handful of studies correlating FAK, Src and PXN with clinicopathological parameters, while the few studies available comparing primary colorectal tumors and liver metastatic sites have provided conflicting results. Ayaki et al [34]. in a small series of patients (n=10), found significantly elevated FAK levels in primary tumors compared to normal mucosa and significantly elevated FAK levels in primary tumors compared to liver metastases. PXN was found significantly elevated in primary tumors compared to normal mucosa, while there was no significant difference compared to liver metastases [34]. Elevated FAK protein levels in both primary CRCs and liver metastases compared with normal colorectal mucosa were measured, while FAK levels were equivalent in the matched CRCs and metastases (n=24) [35]. In fact, FAK mRNA copies were significantly higher in primary tumors than in normal colorectal mucosa. In the same study, in an unmatched liver metastases group, immunohistochemistry demonstrated high FAK expression in the great majority of the samples (89%), while FAK mRNA copies in the same group were significantly higher than FAK mRNA copies in the primary tumors. In a previous study of our group [36] in 80 CRC patients, all tumors were positive for FAK expression compared to normal colonic mucosa where no expression was observed, while in 32 (40%) cases FAK was overexpressed. No significant differences in FAK overexpression were found as far as tumor location, histological grade, stage and the presence or absence of lymphatic invasion is concerned. No association was also found between FAK overexpression and age, gender and Ki-67 positivity. Amongst all the aforementioned parameters, including FAK overexpression, only stage was of any prognostic significance. There was also no statistical difference in the survival rate between patients overexpressing and not overexpressing FAK. This was the first study to correlate FAK expression with clinicopathological parameters [36]. De Heer et al. [37] in a more recent study found that FAK expression was associated with shorter time to recurrence in a near significant level, while Src expression was associated with significantly shorter time to recurrence. On the other hand, PXN expression was not associated with tumor recurrence. Since FAK and Src act as a protein complex, the researchers compared and found that high combined FAK and Src expression was highly significantly associated with shorter time to recurrence [37]. FAK, Src and PXN were found equal in the corresponding liver metastases [38]. The aforementioned studies are characterized by diversity in methodology, definitions of expression and overexpression.

Since metastases are the main cause of death in many malignancies, including CRC, there has been a growing scientific interest around the dif-

ferences between primary and metastatic tumor sites. Many theories have been examined in the past years. It has been suggested that a creation of a "premetastatic niche" is required in the distant site before the establishment of the first tumor cells in order to form metastases . This premetastatic niche is probably a result of endocrine and paracrine signaling networks, which could be affected by therapeutic agents [39-41]. According to the "seed and soil hypothesis" [42] the site of a metastasis is not only influenced by the tumor cell (the seed) but also by the target organ (the soil). In this aspect research has been also focusing on the microenvironment around the metastatic site. It has been shown that non-tumor cells around the metastases differ from normal tissue cells of the target organ [43]. This "tumor stroma", consisting of ECM, fibroblasts, immune and inflammatory cells, resembles an active wound which continuously heals and remodels itself with unceasing angiogenesis and cell proliferation. Tumor cells secrete growth factors and cytokines which activate and recruit fibroblasts and inflammatory cells to the tumor. The latter, in combination with tumor cells, secrete growth factors, ECM components and proteinases that further remodel the tumor stroma [44]. A plethora of cells are engaged in this process, like cancer-associated fibroblasts (CAFs) [45] and immune cells (neutrophils, macrophages, mast cells, lymphocytes. For example, tumor-associated macrophages (TAMs) aid tumor cell intravasation [46] and help in the formation of the premetastatic niche [39]. High infiltration with TAMs has been correlated with worse prognosis [47]. Tumors that secrete factors like osteopontin (OPN) have the ability to activate and mobilize bone marrow-derived cells, which in turn promote primary and metastatic tumor expansion [48]. Other factors, like anti-angiogenic factors, secreted also by primary tumors, may hamper metastasis formation [49]. This translates to a variety of effects that primary tumors can have in the formation and growth of metastases. In the genetic setting, it has been suggested by some researchers that loss of heterozygosity (LOH) of genes like tumor suppressors TP53 and PTEN can influence epithelial-stromal interaction in carcinogenesis [50-52], while others failed to reveal such relevance [43,53]. In another study it was reported that two cell lines derived from the same tumor showed different metastatic potential and properties under certain microenvironment conditions, indicating that the tumor microenvironment is an important factor in the metastatic process [54].

We showed in our study that increased FAK expression in the primary tumors was significantly correlated with high tumor grade and advanced disease stage, while it was also correlated with patient survival at both univariate and multivariate level. Increased Src expression in primary tumors was correlated with positive lymph nodes, while high p-PXN expression with high grade tumors. These results are in line with the relevant literature. A rather interesting finding could be that FAK, Src and p-PXN were found significantly reduced in liver metastases compared to the corresponding primary tumors. At the same time similar proliferation rate of malignant cells was noted in primary and metastatic liver sites. It has been previously shown that the phosphoproteomic status of many protein kinases was entirely different in liver metastases compared to primary colorectal tumors, indicating different signaling pathways which suggest a possible microenvironment effect [55]. Since metastases are the main cause of death in CRC patients, there has been growing interest in elucidating the pathways leading to their creation, while increasing resources have been allocated in discovering therapeutic agents. The relatively new findings around the tumor microenvironment and premetastatic niche could make them a prime target for anti-cancer therapy. The question remains, can the same agents used against primary tumors be as effective against metastases? From the aforementioned mechanisms we could conclude that the formation of the premetastatic niche and metastasis itself is a rather complex procedure, with no clear starting point in the context of a time frame [56]. The premetastatic niche could be formed long before the formation of micro- and macro-metastases, while metastatic cells could remain dormant for long periods of time [57]. Molecules implicated in these procedures, like FAK, Src and p-PXN, could be found up- or downregulated. The decreased expression of the above in liver metastases could either mean that they are essential for the dissemination of the primary tumor but not as important in the formation of metastases or that they have an "early role" in the metastatic process followed by their downregulation. It has previously been shown that cultured liver metastasis-derived CRC cells displayed weaker migratory properties than those of the primary tumor and that the motility-related protein (MRP-1/CD-9) was downregulated in liver metastases [58]. In other studies liver metastasis exhibited significantly higher apoptotic and Ki-67 labeling indices than the primary lesions [59,60]. Decreases in FAK levels resulted in reduced cell motility [61] and increased apoptosis [62,63]. On the contrary FAK overexpression resulted in increased cell motility [64], while it suppressed apoptosis [65]. Thus

a possible FAK downregulation in the metastatic site could be explained as decreased cell motility while it favors apoptosis and proliferation [34].

To our knowledge this is the first time the entire FAK-Src-PXN axis has been studied in a relatively large series of patients, with findings which could be translated that treatments aimed at those molecules cannot influence the course of already established metastases. More research is strongly recommended around molecules implicated in metastasis formation, as well as therapeutic agents targeted in these processes.

Conflict of interests

The authors declare no confict of interests.

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