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Original article

Expression levels of the focal adhesion-associated proteins paxillin and p130^{CAS} in canine and feline mammary tumors

Antonio SCIBELLI[a], Danila D'ANGELO[a], Alessandra PELAGALLI[a], Simona TAFURI[b], Luigi AVALLONE[a], Rossella DELLA MORTE[a]*, Norma STAIANO[a]

[a] Dipartimento di Strutture, Funzioni e Tecnologie Biologiche, Università di Napoli Federico II, Via Delpino 1, 80137 Napoli, Italy
[b] Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Napoli, Italy

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Abstract – Paxillin and p130^{CAS} are two adaptor proteins localized at the focal adhesions which play an important role in cell signaling, cell motility and oncogenic transformation. In this study we evaluated the levels of paxillin and p130^{CAS} in feline and canine mammary tumor tissues at different stages of malignancy. The results obtained by Western blotting analysis showed no significant differences in the amounts of paxillin and p130^{CAS} between normal and non-invasive tumor tissues. By contrast, mammary tumor tissues with the invasive phenotype showed lower levels of paxillin $P < 0.01$ and higher levels of p130^{CAS} $P < 0.001$ than normal tissues. The decrease $P < 0.001$ of the amount of paxillin and the increase $P < 0.001$ of p130^{CAS} levels were correlated with the progression stage of malignancy. Since paxillin and p130^{CAS} are involved in regulating cell migration, our results suggest that low levels of paxillin together with high levels of p130^{CAS} expression may cause certain breast cancers to be more motile and possibly more aggressive. Thus, both paxillin and p130^{CAS} may represent useful prognosticators of feline and canine breast cancer malignancy.

paxillin / p130^{CAS} / mammary tumor / cat / dog

1. INTRODUCTION

The mammary gland is one of the most common sites of tumor development in dogs and cats [14]. Approximately 50% of canine mammary tumors are malignant and the most common malignant tumors are solid carcinomas and adenocarcinomas. Most tumors occur in dogs between 8 and 10 years of age. Mammary cancer occurs much less frequently in cats than in dogs, but when it does occur it is often malignant and difficult to treat. Mammary cancer is likely to strike 1 in 4000 cats. While this is about half the rate as in dogs, when cats develop mammary tumor it is often fatal. Any adult female cat can develop mammary cancer, but the median age is usually

* Correspondence and reprints
Tel.: (39) 081 5644239; fax: (39) 081 5644230; e-mail: rosdella@unina.it

10–14 years. Eighty-five percent of mammary tumors in cats are malignant adenocarcinomas. Other mammary tumors in cats include duct papillomas, sarcomas and adenomas.

Whereas oncology research has been much developed in humans and advanced molecular diagnostic methodologies are largely available in human medicine, the methodologies employed in veterinary oncology for diagnostic purposes are definitely inadequate. For diagnosis and prognosis of mammary tumors in dogs and cats, fine needle aspiration biopsies and histological diagnosis are used. However, there is an extreme histological heterogeneity among mammary gland tumors and it is not uncommon for fine needle aspiration biopsies to yield cells with characteristics of inflammation as the dominant finding rather than characteristics of malignancy. Thus, there is a need to develop new molecular diagnostic strategies for the diagnosis and prognosis of mammary tumors in dogs and cats.

Invasion into surrounding tissues is a prominent phenotype of cancer cells [18]. Regulatory mechanisms of cell motility is critical in this process. The formation of focal adhesions is a regulated process involved in cellular movement. Focal adhesions are structures which form at the site of interaction between the cell actin cytoskeleton and the extracellular matrix (ECM). The focal adhesions contain multiple proteins such as vinculin, talin and tensin. Other proteins localised at focal adhesions include kinases, phospholipases, and proteases. Focal adhesion proteins are key regulators of signal transduction events [5]. The actin cytoskeleton needs to be dynamic for cell shape change alterations in cell contacts and cell motility. The deregulation of cytoskeletal function contributes to the transformation [27]. In normal cells, the cytoskeleton is very stable and there is little movement of the cells. In cells which have become cancerous, the cytoskeleton is disrupted in such a way as to increase cell motility.

Paxillin is a focal adhesion-associated adaptor protein which plays a key role in cell spreading and motility [23]. The protein was originally identified as a substrate for the non-receptor tyrosine kinase oncogene $pp60^{v\text{-}src}$ in Rous sarcoma virus transformed fibroblasts [6]. The human paxillin cDNA was cloned using antibody expression cloning in a K562 cDNA library followed by the polymerase chain reaction and hybridisation strategies [20]. The N-terminal domain of paxillin contains five copies of a peptide sequence, called the LD motif, which are known to function as binding sites for other proteins. The C-terminal half of paxillin is comprised of four LIM domains, which are zinc-binding structures resembling a double zinc finger domain. The focal adhesion targeting sequence of paxillin is located in the C-terminal half of the protein. Paxillin is ubiquitously expressed, even though in variable amounts, with low levels in platelets and neuronal cells. It is highly conserved among various species: the homology between human and chicken predicted amino acids is about 90% with almost complete identity in the LIM domains [22].

Paxillin interacts with several other focal adhesion proteins such as talin, tensin, and vinculin, as well as with important signal transduction intermediates. The tyrosine kinases $p125^{FAK}$, RAFTK and Csk, a negative regulator of $p60^{src}$, are but a few proteins which interact with paxillin at focal adhesions [25].

$p130^{CAS}$ is another focal adhesion-associated protein which has been suggested to play an important role in cell migration [2]. $p130^{CAS}$ was first identified as a phosphotyrosine-containing protein in cells transformed by the oncogenes v-src and v-crk [12]. A cDNA clone encoding $p130^{CAS}$ was isolated in 1994 [19] and its predicted domain structure suggested that it functions as an adapter or scaffolding

protein. $p130^{CAS}$ contains an Src-homology 3 (SH3) domain as well as multiple phosphotyrosine motifs for binding of a variety of SH2 domains [15, 19]. It is phosphorylated upon cell adhesion in a $p125^{FAK}$-$p60^{src}$-dependent manner [17, 26]. $p130^{CAS}$ association with $p125^{FAK}$ has been proposed to initiate a signal transduction pathway to activate Erks [24]. Interestingly, activation of Erks has been shown to regulate cell migration on ECM by directly phosphorylating a cytoplasmic target myosin light chain kinase [9]. However, pathways other than those involving Erks may also be involved in the induction of cell migration by $p130^{CAS}$ [2, 3, 10].

Even though both paxillin and $p130^{CAS}$ have been shown to have a regulatory role in focal adhesion dynamics and cell movement [16], whether and how the two protein signaling pathways, which have been studied in cultured cells, relate to signaling in the complex organization of individual tissues has not yet been established.

In this work we evaluated the expression levels of paxillin, $p130^{CAS}$ and other focal adhesion-associated proteins (talin, vinculin, α-actinin) in mammary tumor tissues from cats and dogs. The expression levels of the two proteins was correlated to the progression stage of malignancy.

2. MATERIALS AND METHODS

2.1. Antibodies and chemicals

Rabbit polyclonal anti-paxillin IgG (H-114) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); mouse monoclonal anti-paxillin IgG (clones 349, 177, 165), and monoclonal anti-$p130^{CAS}$ IgG (clone 21) were purchased from Transduction Laboratories (Lexington, KY, USA); mouse monoclonal anti-vinculin IgG (clone VIN-11-5), anti-talin IgG (clone 8D4), anti-α-actinin IgM (clone BM-75.2) and horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit Ig, aprotinin, bovine serum albumin (BSA), leupeptin, pepstatin from Sigma (St. Louis, MO, USA).

2.2. Tissue sampling and processing

Mammary tumor tissues were obtained from dogs and cats undergoing surgical resection at the Department of Veterinary Clinical Sciences of the University of Napoli Federico II (Italy). The animals were between 6 and 14 years old. Tumors were classified by the criteria of the World Health Organization [7] and were graded as being well, moderately or poorly differentiated (grades 1 to 3, respectively) on the basis of the following parameters: degree of tubule formation, hyperchromatism and number of mitoses, and irregularity of size, shape and staining of the nuclei. The samples consisted of five canine and five feline mammary adenomas, five canine condromas of the mammary gland, five canine mammary adenocarcinomas at grade 1, five canine mammary adenocarcinomas at grade 2, five canine mammary adenocarcinomas at grade 3 and five feline solid carcinomas of the breast. Adjacent normal mammary specimens were obtained from the sites ~5–10 cm apart from the primary tumors. Tissue samples were divided into two parts: one part was fixed in formalin for histological routine diagnosis; another part of the fresh material was perfused thoroughly with cold 0.9% NaCl and homogenized by an Ultraturrax L-407 at 4 °C with 5 mL/1.5 g tissue of buffer containing 10 mM Tris-HCl pH 7.4, 10 mM EDTA, 5 mM dithiotreitol, 10% glycerol, 0.1 U trypsin per mL aprotinin, 10 μg/mL leupeptin and 4 μM pepstatin. Homogenates were divided into small aliquots and stored at –80 °C until use.

The amount of total proteins in each sample was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Electrophoresis and Western blots

Samples containing equal amounts of proteins were boiled for 5 min in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol) and run on 7.5% SDS/polyacrylamide gels. Following electrophoresis, the samples were transferred to nitrocellulose using a Mini Trans-Blot apparatus (Bio-Rad) according to the manufacturer's instructions, blocked for 1 h at 42 °C in Tris/NaCl/P_i containing 5% BSA, and washed with Tris/NaCl/P_i/Tween (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% Tween-20). The blots were incubated for 2 h with anti-paxillin Ig (1:10000) or anti-p130^{CAS} (1:1000) or anti-vinculin (1:500) or anti α-actinin (1:500). After incubation the filters were washed three times with Tris/NaCl/P_i/Tween and incubated for 2 h with peroxidase conjugated anti-(mouse IgG) Ig diluted 1:3000 in Tris/NaCl/P_i, 1% BSA and washed three times with Tris/NaCl/P_i/Tween. The proteins were visualized by an ECL chemiluminescence kit (Amersham Corp., Little Chalfont, UK). The blots were stripped and reprobed against anti-talin antibody (1:500) to confirm equal loading of proteins in each lane. Protein expression levels were quantitatively estimated by densitometry using a Discover Pharmacia scanner equipped with a sun spark classic densitometric workstation. The focal adhesion-associated protein concentrations were normalized to the talin level and expressed as the densitometric ratio.

2.4. Statistical analysis

The paired Student t test was used for statistical analysis and $P < 0.05$ was considered to indicate a significant difference.

3. RESULTS

Mammary tissue samples obtained from cats were homogenized, loaded onto a SDS-polyacrylamide gel and immunoblotted with paxillin, p130^{CAS}, talin, vinculin and α-actinin antibodies. No difference in the amount of paxillin and p130^{CAS} was detected in normal and tumor tissues from cats affected by breast adenoma (Fig. 1, panels A and B, upper blots, lanes 1 and 2). Conversely, a concomitant decrease of the amount of paxillin and increase of p130^{CAS} as compared to normal tissues of the same animal was observed in tumor tissues from cats affected by breast solid carcinoma (Fig. 1, panels A and B, upper blots, lanes 3 and 4). The blots in A and B were stripped and reprobed with an anti-talin antibody to be sure of equal loading in all lanes (Fig. 1, panels A and B, lower blots). No difference in the amount of the other focal adhesion-associated proteins vinculin and α-actinin was detected in tumor tissues as compared to normal tissues from the same animal (Fig. 1, panel C). Similar results were obtained from five samples of feline breast solid carcinomas derived from different animals.

The degrees of paxillin reduction, as compared to normal mammary tissues derived from the same animal, were slightly varied among tumor tissues from different animals. Statistical analysis of paxillin/talin densitometric ratios showed that average paxillin expression levels were significantly lower ($P < 0.01$) in samples of feline breast solid carcinoma than in adjacent normal mammary tissues (Fig. 2, panel A).

The degrees of p130^{CAS} increase, as compared to normal mammary tissues derived from the same animal, were slightly varied among tumor tissues from different animals. Statistical analysis of p130^{CAS}/talin densitometric ratios showed that average p130^{CAS} expression levels were significantly higher ($P < 0.001$) in samples of feline breast solid carcinoma than in adjacent normal mammary tissues (Fig. 2, panel B).

Mammary tissue samples obtained from dogs were homogenized, loaded onto a

Figure 1. Homogenized samples from feline breast adenoma (lane 2), feline breast solid carcinoma (lane 4) and normal mammary tissues of the same animal (lanes 1 and 3) were electrophorized on 7.5% SDS-PAGE and analysed by Western blot with monoclonal mouse anti-paxillin IgG (panel A, upper blot), monoclonal mouse anti-p130^{CAS} IgG (panel B, upper blot), monoclonal mouse anti-α-actinin and monoclonal mouse anti-vinculin (panel C). The blots in panels A and B were stripped and reprobed against anti-talin antibody (lower blots). Molecular mass markers are indicated on the left.

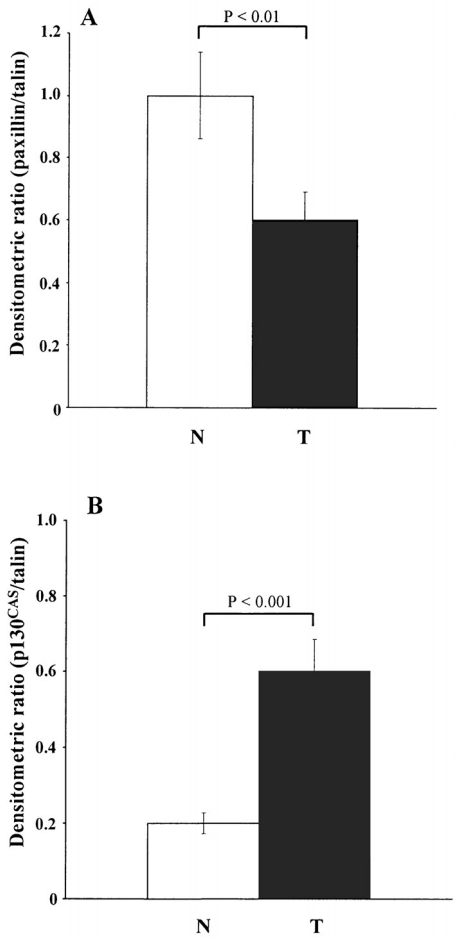

Figure 2. A comparison of average paxillin (panel A) and p130^{CAS} (panel B) expression levels in feline breast solid carcinoma (T) and adjacent normal mammary tissues (N). Data represent the mean ± S.D.

SDS-polyacrylamide gel and immunoblotted with paxillin, p130^{CAS}, talin, vinculin and α-actinin antibodies. No difference in the amount of paxillin and p130^{CAS} as well as of the other cytoskeletal proteins vinculin and α-actinin were observed between normal and tumor tissues from dogs affected by breast adenoma or breast condroma (Fig. 3, panels A and B upper blots, panel C). The blots in A and B were

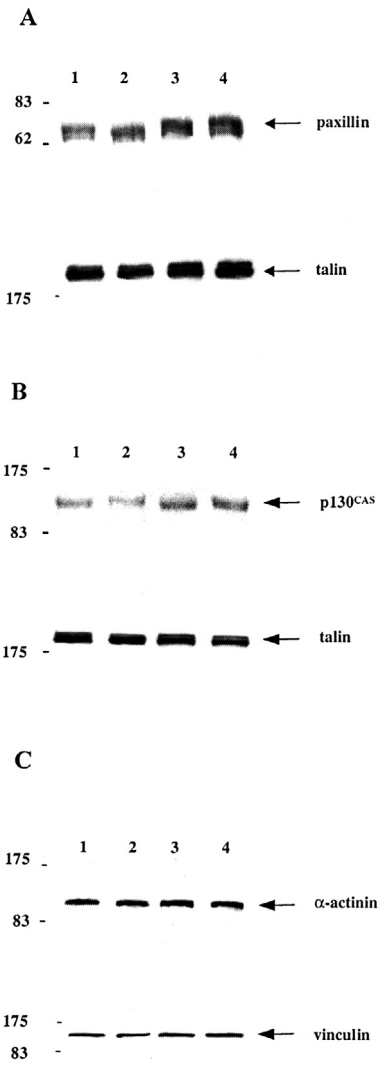

Figure 3. Homogenized samples from canine breast adenoma (lane 2), canine breast condroma (lane 4) and normal mammary tissues of the same animal (lanes 1 and 3) were electrophorized on 7.5% SDS-PAGE and analyzed by Western blot with monoclonal mouse anti-paxillin IgG (panel A, upper blot), monoclonal mouse anti-p130^{CAS} IgG (panel B, upper blot), monoclonal mouse anti-α-actinin and monoclonal mouse anti-vinculin (panel C). The blots in panels A and B were stripped and reprobed against anti-talin antibody (lower blots). Molecular mass markers are indicated on the left.

stripped and reprobed with an anti-talin antibody to be sure of equal loading in all lanes (Fig. 3, panels A and B, lower blots). Similar results were obtained in at least five experiments of identical design performed by using mammary tissues from different animals.

Next, the expression levels of focal adhesion-associated proteins in sample tissues from dogs affected by adenocarcinoma of grade 1, grade 2 and grade 3 were analysed. A decrease of the amount of paxillin and an increase of p130^{CAS} levels were detected in tumor tissues as compared to normal tissues (Fig. 4, panels A and B, upper blots). The decrease of paxillin expression levels and the concomitant increase of p130^{CAS} expression levels correlated with the tumor grading. The blots in A and B were stripped and reprobed with an anti-talin antibody to be sure of equal loading in all lanes (Fig. 4, panels A and B, lower blots). Vinculin and α-actinin levels were the same in tumor tissues and respective normal tissues (Fig. 4, panel C). Similar results were obtained from five samples of each type of tumor derived from different animals.

The degrees of paxillin reduction, as compared to normal mammary tissues derived from the same animal, were slightly varied among tumor tissues from different animals. An almost complete disappearance of paxillin was always observed in all five samples of canine mammary tumors of grade 3. Statistical analysis of paxillin/talin densitometric ratios showed that average paxillin expression levels were significantly lower ($P < 0.001$) in samples of canine breast adenocarcinoma than in adjacent normal mammary tissues and the decrease of the amount of paxillin was significantly correlated with the progression stage of malignancy (Fig. 5, panel A).

The degrees of p130^{CAS} increase, as compared to normal mammary tissues derived from the same animal, were slightly varied among tumor tissues from

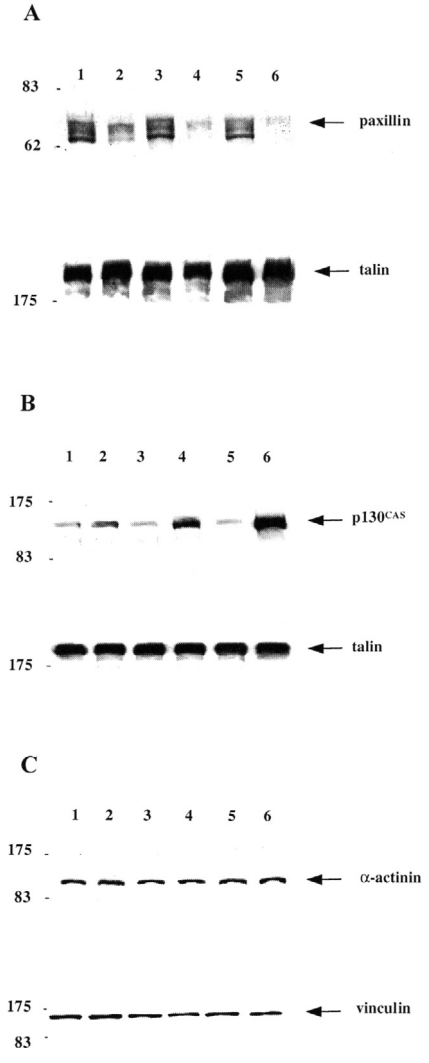

Figure 4. Homogenized samples from canine breast adenocarcinoma at grade 1 (lane 2), canine breast adenocarcinoma at grade 2 (lane 4), canine breast adenocarcinoma at grade 3 (lane 6) and normal mammary tissues of the same animal (lanes 1, 3, 5) were electrophorized on 7.5% SDS-PAGE and analyzed by Western blot with monoclonal mouse anti-paxillin IgG (panel A, upper blot), monoclonal mouse anti-p130^{CAS} IgG (panel B, upper blot), monoclonal mouse anti-α-actinin and monoclonal mouse anti-vinculin (panel C). The blots in panels A and B were stripped and reprobed against anti-talin antibody (lower blots). Molecular mass markers are indicated on the left.

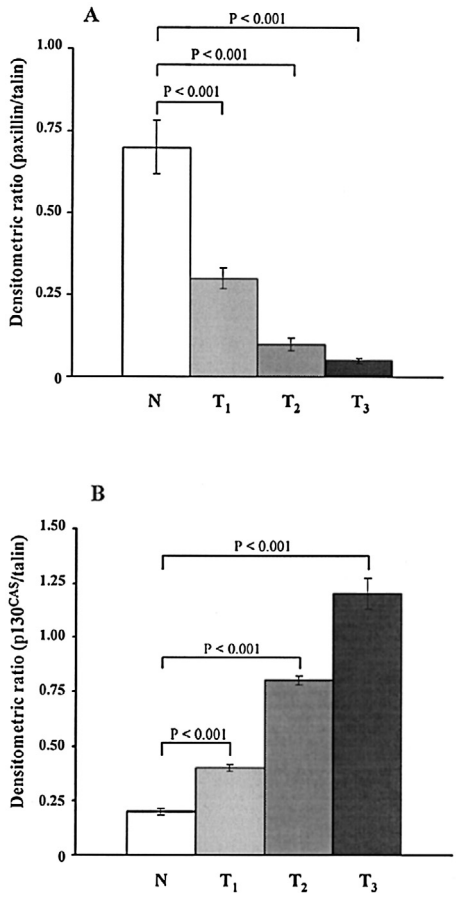

Figure 5. A comparison of average paxillin (panel A) and p130^{CAS} (panel B) expression levels in canine breast adenocarcinomas at grade 1 (T_1), grade 2 (T_2), grade 3 (T_3), and canine adjacent normal mammary tissues (N). Data represent the mean ± S.D.

different animals. Statistical analysis of p130^{CAS}/talin densitometric ratios showed that average p130^{CAS} expression levels were significantly higher ($P < 0.001$) in samples of canine breast adenocarcinoma than in adjacent normal mammary tissues and the increase of p130^{CAS} levels was significantly correlated with the progression stage of malignancy (Fig. 5, panel B).

Experiments performed by using different clones of monoclonal or polyclonal

anti-paxillin antibodies yielded similar results, thus indicating that a possible tumor-specific modification of paxillin at or near sites of the epitope recognition is unlikely to be responsible for the reduced detection in both canine and feline breast cancers.

4. DISCUSSION

In this study we measured the expression levels of five focal adhesion-associated proteins (paxillin, $p130^{CAS}$, talin, vinculin, α-actinin) in different types of feline and canine mammary tumor tissues. No significant differences in the protein expression levels between normal and non-invasive tumor tissues were observed. By contrast, mammary tumor tissues with an invasive phenotype showed lower levels of paxillin and higher levels of $p130^{CAS}$ than normal tissues. The decrease of the amount of paxillin and the increase of $p130^{CAS}$ levels correlated with the progression stage of malignancy.

Further studies are required to establish whether the observed down-regulation of paxillin in tumor tissues is due to a proteolytic degradation of the protein or to a down-regulation of paxillin mRNA expression. Northern blot analysis was not performed due to the limited availability of the tissues. Moreover, the observed decrease of the paxillin amount in tumor tissues as compared to normal tissues refers to the total amount of cellular paxillin; it would be interesting to investigate whether a more pronounced decrease of the amount of paxillin localized at focal adhesions would occur rather than a decrease of the cytosolic localized protein levels. Finally, whether the observed up-regulation of $p130^{CAS}$ expression in tumor tissues could be due to increased synthesis of newly transcribed mRNA and/or to the enhanced stability of $p130^{CAS}$ mRNA should be evaluated.

The amount of paxillin has been shown to be significantly reduced during mitosis of the cell cycle, whereas the expression levels of other focal adhesion proteins such as talin, vinculin and $p125^{FAK}$ remained unchanged [28]. Low levels or the absence of only paxillin expression have also been demonstrated in certain human lung cancers [21] and liver metastasis [1]. Cell motility, as assessed by migration membrane ruffling and the formation of lamellipodia and filopodia, has been shown to be inhibited by paxillin overexpression in lung cancer cell lines [21]. Many studies using different approaches to address the role of paxillin in the cell have produced the same conclusion that paxillin controls cell spreading and motility [23]. Overexpression of wild type paxillin inhibits the ability of lysophosphatidic acid stimulated MM-1 rat hepatoma tumor cells to invade a monolayer of primary mesothelial cells [29]. Paxillin has also been implicated in the control of cell spreading and motility by the α_4 integrin subunit [11], since an α_4 mutant defective for paxillin binding failed to block cell spreading.

$p130^{CAS}$ appears to function as an important "molecular switch" for the induction of migration signals via its binding to the SH2/SH3-adapter protein Crk [10]. $p130^{CAS}$ overexpression enhances cell migration of COS and FG-M pancreatic carcinoma cells plated on vitronectin. Expression of a substrate binding domain deletion of $p130^{CAS}$ that is deficient for Crk binding inhibits this effect [10]. $p130^{CAS}$-Crk signaling also appears to be important for haptotaxis involving ECM components other than vitronectin. For example, cells expressing a mutant of the α_7 integrin subunit exhibit diminished $p130^{CAS}$ phosphorylation and $p130^{CAS}$-Crk association resulting in a decreased migration on laminin [13]. $p130^{CAS}$-deficient fibroblasts show decreased haptotaxis toward fibronectin, a decreased ability to migrate into the gap in a wound healing assay, and a decreased basal

and serum-induced invasion through a 3-dimensional collagen matrix [4, 8].

Finally, the close functional relationship between $p125^{FAK}$ and $p130^{CAS}$ also contributes to the role of $p130^{CAS}$ in cell migration. Overexpression of $p125^{FAK}$ in CHO cells enhances fibronectin-mediated haptotaxis and this effect can be inhibited by co-expression of the $p130^{CAS}$ SH3 domain [3]. Under these conditions the $p130^{CAS}$ SH3 domain competes with endogenous $p130^{CAS}$ in binding to $p125^{FAK}$, thus inhibiting functional interactions between $p125^{FAK}$ and full-length $p130^{CAS}$.

Metastasising cells tend not to form clusters and exhibit cell-to-cell contacts, which are the opposite of normally functioning cells. Low levels of paxillin and high levels of $p130^{CAS}$ may confer to cancer cells an advantage in migration by interfering with the attachment with the extracellular matrix, and this may involve blocking or sequestering signaling molecules or interfering with signal transduction from focal adhesions. Thus, the observation that paxillin levels are reduced and concomitantly $p130^{CAS}$ levels are increased in malignant mammary tumors at high grades of progression and are therefore more aggressive, may lead to further insights into the role of paxillin and $p130^{CAS}$ in normal and transformed cells.

The important finding emerging from this study is the presence of low levels of paxillin and high levels of $p130^{CAS}$ in invasive feline and canine mammary tumors. Even though additional studies using a larger number of clinical samples are needed to validate these preliminary findings, to the best of our knowledge this is the first report demonstrating a significant correlation between the expression levels of paxillin and $p130^{CAS}$ and the mammary tumor malignancy stage. Thus, paxillin and $p130^{CAS}$ may represent useful prognosticators of feline and canine breast cancer malignancy. Whether the two focal adhesion proteins could be used as prognosticators of human breast cancer malignancy would be intriguing to establish.

Our results may provide the basis for developing new diagnostic and therapeutic strategies in veterinary oncology. At the moment we are investigating whether paxillin and $p130^{CAS}$ expression is down- or up-regulated in domestic animal cancers other than mammary tumors.

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