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A preliminary investigation of the role of the transcription co-activators YAP/TAZ of the Hippo signalling pathway in canine and feline mammary tumours

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ABSTRACT

Breast cancer is the most common cancer in women worldwide. Cancer metastases are responsible for the high mortality rate. A small but distinct subset of cells, cancer stem cells (CSCs), have the capacity to self-renew, initiate tumour formation, and develop metastases. The CSC content in human breast cancer correlates with the Hippo tumour suppressor signalling pathway. Specifically, the activity of YAP/TAZ, transcription co-activators of the Hippo pathway, sustains the self-renewal and tumour-initiation capacities of CSCs. Little is known about YAP/TAZ in canine and feline mammary tumours, which are very common tumours. The preliminary aim of the study was to investigate the expression of YAP/TAZ in canine and feline mammary tumours by Western blot and immunohistochemistry.

Increased cytoplasmic and nuclear expression of YAP/TAZ was observed in all carcinomas compared to normal tissues, indicating neoplastic deregulation of the Hippo pathway. Nuclear expression significantly increased in grade III (high grade carcinomas) compared to grade I (low grade carcinomas) tumours, suggesting that YAP/TAZ play a role in the increased aggressiveness of these tumours. Moreover, different scoring systems for immunohistochemical analyses were compared and the H index and the Allred scores were the most significant. In conclusion, YAP/TAZ are expressed in aggressive canine and feline mammary tumours as reported in some human cancers. Further studies might better elucidate the role of the Hippo pathway in prognosis and as a target for new therapies. In addition, tumours in dogs and cats may be a useful model to study this pathway.

Introduction

Human breast cancer (HBC) accounts for 23% of all cancers worldwide (Jemal et al., 2011; Siegel et al., 2013). Mammary gland tumours (MTs) are the third most common cancer in intact cats, and 80–90% are malignant and aggressive carcinomas (Withrow and MacEwen, 2012; Morris, 2013; Zappulli et al., 2015). MTs are also the most common neoplasm in intact bitches; 20% are malignant and of these 20% are likely to metastasize (Misdorp et al., 1999; Perez Alenza et al., 2000; Peña et al., 2013).

Recent studies have demonstrated that in some tumours, such as HBC, a small but distinct subset of cells – known as cancer stem cells (CSCs) – has the capacity to self-renew and initiate tumour formation (Rios et al., 2014; Visvader and Stingl, 2014). It has been suggested that CSCs have the ability to detach from the primary site and develop metastases, the major cause of death in cancer patients. CSCs are also highly resistant to conventional therapies and are capable of escaping targeted treatments. For these reasons, an understanding of CSC regulation could provide new insights into the treatment of cancer (Liu and Wicha, 2010; Guo, 2014).

Hippo is a highly conserved tumour-suppressor signalling pathway that plays a key role in the regulation of organ size (Mo et al., 2014). Its deregulation results in tissue overgrowth and tumorigenesis in humans (Hong and Guan, 2012; Jeong et al., 2013; Piccolo et al., 2013; Vermeulen, 2013; Han et al., 2014; Liang et al., 2014; Sanchez and Aplin, 2014). In HBC, the Hippo pathway was recently found to correlate with CSCs in high-grade tumours (Cordenonsi et al., 2011). Primarily, the activity of YAP/TAZ, paralog

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downstream transcription co-activators of the Hippo pathway, sustains self-renewal and the tumour-initiation capacities of breast stem cells. Moreover, YAP/TAZ are overexpressed in many human cancers and promote cell proliferation and epithelial to mesenchymal transition (EMT). Therefore, the Hippo pathway has been considered as a potential target for new therapies (Cordenonsi et al., 2011; Hao et al., 2014).

Little is known about YAP/TAZ and the Hippo pathway in canine (CMCs) and feline mammary carcinomas (FMCs). Therefore, in this preliminary study, our aims were to evaluate the expression of the YAP/TAZ proteins in normal and neoplastic feline and canine mammary tissues (44 carcinomas) and to compare the most commonly used scoring systems for immunohistochemistry (IHC) (Peña et al., 2014).

Materials and methods

Tissues and samples

Archived feline and canine mammary tissues were selected from cases submitted to our Institution (Padua, Italy) between 2002 and 2013. All tissue samples were obtained by surgical resection and routinely processed for histology. Classification of CMCs was performed using the revised World Health Organization (WHO) classification system (Goldschmidt et al., 2011) and graded using a recent system (Peña et al., 2013). FMCs were classified and graded according to the most commonly used systems (Castagnaro et al., 1998; Misdorp et al., 1999). Forty-four samples were included in the study (Table 1). The specific subtypes included: feline simple carcinomas grade I ($n = 6$) (tubular and tubular papillary) and grade III ($n = 6$) (tubular, solid, comedocarcinoma), feline ductal-associated carcinomas ($n = 4$); canine simple tubular grade I ($n = 8$), solid grade III ($n = 7$), complex grade I carcinomas ($n = 6$), and canine carcinomas with malignant myoepithelioma (CCMMs) ($n = 7$). In case of multiple nodules on the same subject, the most malignant one was selected for the analysis.

Feline ductal-associated carcinomas, canine complex carcinomas, and canine carcinomas with malignant myoepithelioma (CCMMs) showed a biphasic nature with both luminal epithelial and basal/myoepithelial cells. The latter manifested a basal morphology in feline ductal-associated carcinomas, a well differentiated myoepithelial morphology in complex carcinomas, and a malignant undifferentiated myoepithelial morphology in CCMMs. Only a luminal epithelial neoplastic population characterised simple tumours.

Protein extraction

Proteins were extracted from RNALater-preserved tissues from two triple-negative HBCs, (TNBCs) (ER-/PR-/HER2-), two CMCs (CCMMs), and two FMCs (STC grade II). Tissues (20 µg) were homogenised using RyboLyser homogeniser (Hybaid) in phosphate buffered saline (PBS). The homogenates were then centrifuged for 10 min at 10,000 *g* and the supernatant was collected in a fresh tube. Lysis buffer (40 mL; 2% sodium dodecyl sulphate (SDS), 10 mM Tris pH 6.8, 10% glycerol) and 2 mL of protease inhibitory cocktail were added for each 100 mL of supernatant obtained after centrifugation. Supernatants were then incubated for 10 min at 4 °C and then 5 min at room temperature after the addition of DNAse.

Nuclear fraction was isolated from RNALater-preserved tissues from the two TNBCs and from two additional FMCs (STC grade III) and two additional CMCs (STC grade I). Tissue was briefly minced with scissor and homogenised in a low-ionic strength medium (10 mM Tris–HCl, pH 7.4, 0.5 mM $MgCl_2$, and protease inhibitors, 100 µM phenylmethylsulfonyl fluoride (PMSF), 0.5 µg/mL leupeptin) using a conical glass homogenizer with a Teflon pestle. The homogenate was diluted with an equal volume of a solution of 10 mM Tris–HCl, 0.5 M sucrose, and 300 mM KCl. The suspension was centrifuged at 10,000 *g* for 20 min to pellet nuclei (Maruyama and MacLennan, 1988). The soluble fraction was collected and nuclear pellet was solubilised in 5% sodium deoxycholate containing protease inhibitors. Protein concentration was determined using bovine serum albumin as standard (Lowry et al., 1951). Protein fractions were stored at –80 °C.

Western blot (WB) analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described using 10% polyacrylamide gels (Sacchetto et al., 2009). After electrophoresis, proteins were transferred onto nitrocellulose. The blots were probed with rabbit polyclonal antibodies anti-WW domain-containing transcription regulator protein 1 (WWTR1) (1:500) (Sigma Aldrich) recognising both the YAP and TAZ human proteins, according to the manufacturer's instructions.

Immunohistochemistry

IHC was conducted using an anti-WWTR1 rabbit polyclonal antibody (Sigma Aldrich). Sections (4 µm) were processed with an automatic immunostainer (BenchMark XT, Ventana Medical Systems). Briefly, the automated protocol included: a high temperature antigen unmasking (60 min with the CC2 reagent); a washing step (8 min with the antibody diluent); the primary antibody incubation (1:100 dilution, 60 min, room temperature); an ultrablock (4 min with the antibody diluent); and haematoxylin counterstaining (4 min). The UltraView Universal DAB detection kit was used. Negative controls omitted the primary antibody, whereas adnexa and epidermis, when present, were used as positive controls. Two TNBCs used in WB were also included in the IHC analysis.

Evaluation of IHC and statistical analysis

A semi-quantitative evaluation was performed by at least two separate operators for all MTs. Cytoplasmic and nuclear positivity was recorded as percentage of positive cells. The nuclear staining intensity was also scored as intense staining (3 points), moderate staining (2 points), poor staining (1 point), and no staining (0 point). Different cell populations (complex carcinomas, CCMMs, ductal-associated tumours) were scored independently. Tumour stroma and – when available on the slide – epidermis, subcutis, and non-neoplastic mammary tissue were also assessed.

To express the final score for each MT and for further statistical analysis only the nuclear staining was considered, and a rough value of positivity for each case was calculated as the sum of all the Intensively, Moderately, and Poorly positive nuclei (IMP%). Different IHC scoring systems were also used as summarised in Table 2.

For the Quick Score (Barnes et al., 1993), the Immunoreactive Score (IRS) scale (Remmele and Stegner, 1987), and the Allred Score (Allred et al., 1998), two values were obtained for each sample to be compared in the statistical analyses. Many samples had positive cells of different staining intensities, but the literature does not indicate how the percentages of positive cells with different intensities should be considered in calculating the scores. In our study, a standard (st) value was

Table 1
Diameter and immunohistochemical nuclear expression of YAP/TAZ in feline and canine mammary carcinomas (FMCs and CMCs) as average score ± standard deviation (SD) according to different scoring systems (IMP%, H, Q, IRS, Allred) calculated as two different values (st, standard, and av, average; see text) and comparison of the two species, and different histological subtypes and grades.

N. of cases		FMCs	CMCs	FMCs				CMCs			
				SCs	DCs	Grade I	Grade III	SCs	Biphasic[a]	Grade I	Grade III
		16	28	12	4	10	6	15	13	21	7
YAP/TAZ IHC scores	Diameter (mm)	10.1 ± 5.1	12 ± 9.8	11.3 ± 5.1*	6.5 ± 2.7*	8.5 ± 5.2*	12.7 ± 3.5*	7.7 ± 6.9**	16.9 ± 10.3**	12.3 ± 10.2	11.1 ± 8.1
	IMP%	65.5 ± 25.3*	79.2 ± 15.3*	77.4 ± 16.6**	29.8 ± 5.1**	53.5 ± 21.9*	85.5 ± 16.3*	80.9 ± 16.5	77.2 ± 13.6	74.1 ± 14.2**	94.7 ± 3.7**
	H score	106 ± 55.8*	134.6 ± 37.4*	129.2 ± 44.8**	36.8 ± 6.4**	74.4 ± 33**	158.8 ± 45.1**	140.1 ± 42.4	128.3 ± 29.3	120.1 ± 30.8**	178.3 ± 14.7**
	Q_{av}	3.4 ± 0.4	3.5 ± 0.3	3.6 ± 0.3**	3.1 ± 1.1**	3.3 ± 0.3*	3.7 ± 0.1*	3.5 ± 0.2	3.5 ± 0.3	3.4 ± 0.3**	3.7 ± 0**
	Q_{st}	4.4 ± 1.5	4.9 ± 0.9	4.9 ± 1.3**	2.8 ± 0.4**	3.6 ± 0.9**	5.7 ± 1.3**	4.9 ± 0.9	4.9 ± 0.9	4.7 ± 0.9*	5.6 ± 0.5*
	IRS_{av}	3.3 ± 0.7	3.5 ± 0.5	3.6 ± 0.6**	2.3 ± 0**	2.9 ± 0.5**	3.9 ± 0.5**	3.6 ± 0.5	3.4 ± 0.6	3.4 ± 0.5**	4 ± 0**
	IRS_{st}	4.5 ± 3.4	4.6 ± 2.5	5.3 ± 3.5**	2 ± 0**	2.6 ± 0.7**	7.7 ± 3.7**	4.8 ± 2.3	4.3 ± 2.6	4 ± 2.3**	6.3 ± 2**
	$Allred_{av}$	4.9 ± 0.4*	5.2 ± 0.4*	5.1 ± 0.3**	4.3 ± 0**	4.7 ± 0.4*	5.3 ± 0.3*	5.3 ± 0.4	5.1 ± 0.3	5.1 ± 0.3**	5.7 ± 0**
	$Allred_{st}$	5.7 ± 1.3*	6.2 ± 0.6*	6.2 ± 1.1**	4.3 ± 0.4**	5.1 ± 0.8*	6.7 ± 1.3*	6.2 ± 0.7	6.2 ± 0.6	6.1 ± 0.6*	6.6 ± 0.5*

SC, simple carcinomas; DCs, ductal-associated carcinomas.
[a] Biphasic CMCs included complex carcinoma and carcinoma with malignant myoepithelioma.
* $P < 0.05$; ** $P < 0.01$.