

Expression of Myoepithelial Markers p63 and 14-3-3 σ in Canine Mammary Tumours

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ABSTRACT: p63, a p53 homologue nuclear transcription factor was expressed in basal cells of multilayered epithelia and myoepithelial cells of the breast. 14-3-3 σ was a putative tumour suppressor gene, transactivated by p53 in response to DNA damage and immunohistochemically demonstrated that 14-3-3 σ was consistently expressed in the cytoplasmic compartment and occasionally in the nuclei of myoepithelial cells. In the present study, p63 immunoreactivity was restricted to nucleus of myoepithelial cells. Eighty per cent of tumours showed immunopositivity. Mild to moderate staining was noticed. The intensity of staining in carcinomas / carcinosarcomas were mild in 40 per cent, moderate in 25 per cent and intense in 15 per cent. p63 expression was found in 80 per cent of the canine mammary tumours evaluated. Malignant mixed tumours showed 40-80 per cent immunoreactivity. It was 85-95 per cent in spindle cell carcinoma and 70-90 per cent in squamous cell carcinoma. 80-90 per cent p63 immunopositivity was noticed in squamous cell carcinoma. In cystadenoma, no immunoreactivity was observed. 14-3-3 σ immunoreactivity was observed both in the nuclear and cytoplasmic components of myoepithelial cells. 14-3-3 σ immunoreactivity was observed in 95 per cent of the tumours evaluated and immunoreactivity was noticed both in the cytoplasmic and nuclear components. Benign tumours showed 5% expression and malignant mixed tumours showed 25 - 80 per cent 14-3-3 σ immunoreactivity. Squamous cell carcinoma showed 80 per cent immunoreactivity.

KEYWORDS: p63, 14-3-3 σ , Myoepithelial marker, Canine mammary tumour.

I. INTRODUCTION

Mammary gland tumours represented approximately 42 per cent of all tumours and 82 per cent of those arising on the female reproductive organs (Cotchin, 1954). The incidence of mammary carcinomas in canines is three times more than that documented in humans (Sorenmo, 2003). Tumour markers are biochemical indicators of the presence of a tumour. These include cell-surface antigens, cytoplasmic proteins, enzymes and hormones (Kumar *et al.*, 2005). The epithelial cells that constitute the mammary gland are arranged in two layers, the luminal epithelial layer and the basal myoepithelial layer (Deugnier *et al.*, 2002). Myoepithelial cell is the "Cinderella of mammary cell biology" and is a "natural tumour suppressor" (Lakhani and O'Hare, 2001; Deugnier *et al.*, 2002). Myoepithelial cells constitute the basal cell layer of normal mammary epithelia, and their identification is of particular diagnostic value because they are retained in most benign lesions while being lost in malignancy (Barbareschi *et al.*, 2001).

Among the myoepithelial markers, p63, a p53 homologue is a sensitive and highly specific marker of myoepithelial cells in canine mammary tissues and it is the first nuclear marker of myoepithelial cells (Barbareschi *et al.*, 2001; Reis-Filho *et al.*, 2002, 2003; Reis-Filho and Schmitt, 2002; Gama *et al.*, 2003). Hermeking *et al.* (1997) and Laronga *et al.* (2000) reported that 14-3-3 σ was associated with both G1 and G2/M cell-cycle arrest through p53 – dependent transactivation in response to DNA damage. Ferguson *et al.* (2000) reported that 14-3-3 σ might be a tumour suppressor gene, in spite of the rare occurrence of 14-3-3 σ germ-line or somatic mutations in breast neoplasms.

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Myoepithelial cell markers, whose expression is altered during neoplastic transformation of breast epithelial cells, are presently taken up in the study of canine mammary tumours.

II. RELATED WORK

Barbareschi *et al.* (2001), Reis – Filho *et al.* (2002, 2003) Reis – Filho and Schmitt (2002) and Gama *et al.* (2003) reported that p63, a p53 homologue nuclear transcription factor was expressed in basal cells of multilayered epithelia and myoepithelial cells of the breast and also found it as the reliable marker of myoepithelial cells of breast lobules and ducts. It was the first identified nuclear marker of myoepithelial cells. Yaziji *et al.* (2000) and Barbareschi *et al.* (2001) reported the presence of myoepithelial cells as a marker of benign breast lesions while being lost in malignancy.

Ferguson *et al.* (2000), Iwata *et al.* (2000), Suzuki *et al.* (2002), Osada *et al.* (2002) and Umbricht *et al.* (2001) reported that 14-3-3 σ gene was hypermethylated in a proportion of human neoplasm including breast carcinomas. Ferguson *et al.* (2000) reported that 14-3-3 σ expression was lost in approximately 95% of breast carcinomas by methylation – mediated silencing. Umbricht *et al.* (2001) showed that hypermethylation of 14-3-3 σ occurred at an early stage in the progression of invasive breast cancer. Van Hemert *et al.* (2001) and Tzivion *et al.* (2001) reported that 14-3-3 iso forms constituted an important family of highly conserved proteins of 25 - 30 KD that functioned in multiple regulatory process including intracellular signalling, apoptosis and cell cycle control.

Hermeking (2003) reported that 14-3-3 proteins regulated enzyme activity and may act as localization anchors, controlling the subcellular localization of proteins and they can function as adaptors or scaffolds stimulating protein-protein interactions. Simpson *et al.* (2004) reported that 14-3-3 σ was a putative tumour suppressor gene, transactivated by p53 in response to DNA damage and immunohistochemically demonstrated that 14-3-3 σ was consistently expressed in the cytoplasmic compartment and occasionally in the nuclei of myoepithelial cells arranged as a continuous layer around normal ducts and lobular units. Perathoner *et al.* (2005) found 14-3-3 σ as an intracellular, dimeric, phosphoserine binding protein that was expressed in epithelial cells and involved in cancer development and also defined it as a negative regulator of cell cycle progression.

III. MATERIALS AND METHODS

Case selection

From 39 dogs, 63 mammary tissue samples (57 glands were mastectomized and six glands were obtained from two necropsy cases) which were brought to the small Animal Surgical ward of Madras Veterinary College Teaching Hospital and Department of Veterinary Pathology, Madras Veterinary College, TANUVAS, Chennai during September 2005 to June 2006. All samples were fixed in 10% neutral buffered formalin and processed through alcohol and xylol and embedded in paraffin sections. Tissue sections were stained by haematoxylin and eosin (Bancroft *et al.*, 1996) and the tumours were classified as per WHO Classification (Hampe and Misdorp, 1974).

Immunohistochemical examination

Immunohistochemical staining for p63 (Gama *et al.*, 2003) and 14-3-3 σ (Simpson *et al.*, 2004) were done on canine mammary tissue samples.

Tissue sections were labeled for p63 using monoclonal antibody (Bio Genex, USA) and polyclonal antibody for 14-3-3 σ (Santa Cruz Biotechnology, USA). 1-2 μ m thick paraffin embedded tissue sections were prepared on Poly – L – Lysine coated slides and were dewaxed and dehydrated in graded series of alcohol. The sections were then subjected to high temperature antigen retrieval by placing in citrate buffer (pH 6.0) in the microwave for 10 min. After cooling, the sections were washed in Phosphate Buffered Saline (PBS) (pH 7.2 to 7.4) twice for 5 min each. Tissue sections were blocked with peroxidase block (p63) and normal serum (14-3-3 σ) for 10 min. The sections were washed in PBS (pH 7.2 to 7.4) twice for 5 min each. Tissue sections were incubated with primary antibody for 1 h (p63) and 14-3-3 σ (1:50 dilution). The sections were washed in PBS (pH 7.2 to 7.4) twice for 5 min each. Tissue sections were incubated with secondary antibody LSAB secondary antibody kit, Bio Genex (p63) and Vector labs (14-3-3 σ - 1:400) for 45 min. The sections were washed in PBS (pH 7.2 to 7.4) twice for 5

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min each. Tissue sections were incubated with streptavidin for 30 minutes. Tissue sections were washed in PBS (pH 7.2 to 7.4) twice for 5 min each. Tissue sections were immunostained with Diaminobenzidine tetrahydrochloride (DAB) chromogen for 5- 40 min. Tissue sections were washed in distilled water for 3 min. Slides were then counterstained with Harri's Haematoxylin, kept in xylene for 30 sec and then mount in DPX.

Immunoreactivity scoring

p63 positivity was indicated by the presence of distinct brown nuclear staining. The number of immunoreactive cells was assessed semiquantitatively (Gama *et al.*, 2003).

-	=	No stained cells
±	=	Less than 10% positive cells
+	=	10 - 50% positive cells
++	=	More than 50% positive cells

The distribution and intensity of 14-3-3σ staining were evaluated semi - quantitatively (distribution: 1 = <5% of cells stained, 2 = 5 - 25%, 3 = 25-50%, 4=>50%), intensity: 0 = Negative, + = Weak, ++ = Moderate, +++ = Strong (Simpson *et al.*, 2004).

IV. RESULTS

p63 immunoreactivity was restricted to nucleus of myoepithelial cells. Eighty per cent of tumours showed immunopositivity. Mild to moderate staining was noticed. The intensity of staining in carcinomas / carcinosarcomas was mild in 40 per cent, moderate in 25 per cent and intense in 15 per cent. The gradation belonged to 20 per cent (-), 30 per cent (+), (++) 50 percent, Cystadenoma showed negative immunoreactivity but died of other reasons. Tubular adenocarcinoma showed negative, mild or intense staining. Complex tubular adenocarcinoma got intensely (++) stained. One of the papillary cystic adenocarcinoma showed moderate staining while the other showed no staining. Squamous cells carcinoma showed moderate and intense staining. Solid carcinoma showed moderate intensity while spindle cell carcinoma and papillary tubular adenocarcinoma showed mild immunoreactivity respectively. Most of carcinosarcoma (6/7) showed mild intensity (fig a-c).

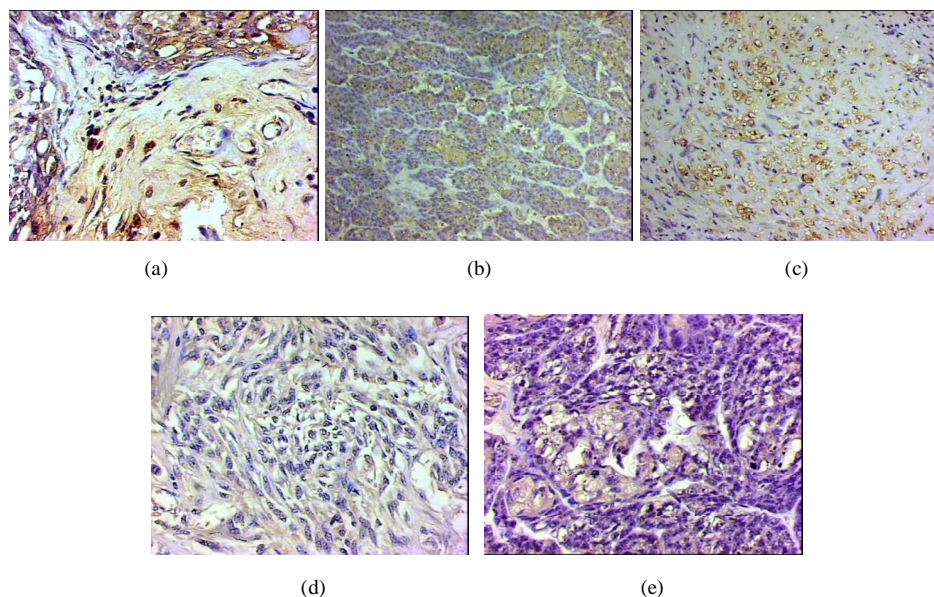


Fig. (a).p63 Carcinosarcoma – intense SABC x400 (b) p63 Squamous cell carcinoma- moderate SABC x 400 (c) p63 Tubular Adenocarcinoma – intense SABC x400 (d) 14-3-3σ Cystic Papillary Adenocarcinoma -weak SABC x 400 (e) 14-3-3σ Squamous cell carcinoma- moderate SABC x 400. SABC – Streptavidin Biotin Complex.

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p63 AND 14-3-3σ IMMUNOREACTIVITY, PER CENT POSITIVITY, GRADING AND SURVIVABILITY IN CANINE MAMMARY TUMOURS

Sl. No.	Type of tumours	p63		14-3-3σ		Survivability		Remarks
		Intensity	Grade	Intensity	Grade	DFS (days)	OS (days)	
1.	Cystadenoma	No staining	-	Weak	1+	125	125	Dead
2.	TAC	No staining	-	Weak	2+	242	242	Alive
3.	TAC	Mild	+	Moderate	3+	0	0	Dead
4.	TAC	Intense	++	Moderate	3+	162	770	Recurrence / metastasis
5.	TAC	No staining	0	No staining	0	108	108	Alive
6.	Complex TAC	Intense	++	Strong	2+	210	570	Recurrence / metastasis
7.	PCAC	No staining	-	Moderate	4+	154	154	Alive
8.	PCAC	Moderate	+	Weak	3+	154	154	Alive
9.	SQCC	Intense	++	Weak	3+	62	62	Dead
10.	SQCC	Moderate	++	Moderate	4+	118	118	Alive
11.	SC	Moderate	++	Moderate	4+	62	62	Dead
12.	SCC	Mild	++	Moderate	4+	32	95	Recurrence / metastasis
13.	PTAC	Mild	+	Weak	1+	263	263	Alive
14.	CS	Mild	+	Weak	3+	182	182	Alive
15.	CS	Moderate	++	Weak	3+	0	0	Dead
16.	CS	Mild	+	Weak	2+	132	132	Alive
17.	CS	Mild	+	Moderate	2+	162	770	Recurrence / metastasis
18.	CS	Mild	++	Moderate	4+	106	106	Alive
19.	CS	Mild	++	Intense	3+	112	112	Alive
20.	CS	Mild	++	Moderate	4+	90	90	Alive

p63 - = No stained cells, + = 10 - 50% positive cells, ++ = More than 50% positive cells.

14-3-3σ 1+ = <5%, 2+ = 5-25%, 3+ = 25-50%, 4+ = >50% of cells stained.

DFS - Disease free survival (Days), OS - Overall survival (Days).

TAC - Tubular adenocarcinoma, PCAC - Papillary cystic adenocarcinoma, SQCC - Squamous cell carcinoma, SC - Solid carcinoma, SCC - Spindle cell carcinoma, PTAC - Papillary tubular adenocarcinoma, CS - Carcinosarcoma.

14-3-3σ immunoreactivity was observed both in the nuclear and cytoplasmic components of myoepithelial cells. Out of 20 tumours 19 (95.00%) were immunopositive. Intensity of staining observed was weak (40%), moderate (40%) and strong (15%). The gradation belong to 1+ = 10 per cent, 2+ = 20 per cent, 3+ = 35 per cent and 4+ = 30 per cent. Weak immuno staining (1 + grade) was noticed in benign tumour. One tubular adenocarcinoma showed no staining, one weak (2+) and 2 moderate (3+). One animal from 3+ grade died. A recurrent case of complex tubular adenocarcinoma with metastasis showed strong immunostaining (2+). One case with papillary cystic adenocarcinoma and another with squamous cell carcinoma showed weak (3+) and moderate (4+) staining respectively. In squamous cell carcinoma, one animal with weak staining died. Solid carcinoma and spindle cell carcinoma showed moderate staining (4+). Death was noticed in one moderately stained solid carcinoma case. Weak and moderate staining was noticed in papillary tubular adenocarcinoma. Among the seven carcinosarcomas, 2 cases were of grade 4, 3 cases of grade 3 and 2 cases of grade 2 (fig d-e).

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V. DISCUSSION

p63 expression was found in 80 per cent of the canine mammary tumours evaluated. Malignant mixed tumours showed 40-80 per cent immunoreactivity. It was 85-95 per cent in spindle cell carcinoma and 70-90 per cent in squamous cell carcinoma. 80-90 per cent p63 immunopositivity was noticed in squamous cell carcinoma. In cystadenoma, no immunoreactivity was observed. Barbareschi *et al.* (2001) reported that in human breast tissue, p63 was preferentially expressed by myoepithelial cells and proposed a close link between myoepithelial and stem or breast progenitor cells. Gama *et al.* (2003) found p63 expression in 91.4 per cent of samples evaluated. In the present study, p63 immunopositivity was observed in 80 per cent of tumours evaluated. p63 expression was restricted to myoepithelial cell nuclei and showed a strong p63 immunoreactivity with a basal cell distribution in squamous cell carcinoma. Barbareschi *et al.* (2001) reported that in cytologic preparations p63 immunoreactivity was a consistent feature of naked nuclei and of a subset of cells surrounding benign epithelial clusters. Reis – Filho *et al.* (2002) found the usefulness of p63 positive myoepithelial cells overlying malignant cell cluster and admixed with malignant cells in differentiating ductal carcinoma *in situ* (DCIS) and invasive carcinoma.

14-3-3 σ immunoreactivity was observed in 95 per cent of the tumours evaluated and immunoreactivity was noticed both in the cytoplasmic and nuclear components. Benign tumours showed 5 per cent expression and malignant mixed tumours showed 25 - 80 per cent 14-3-3 σ immunoreactivity. Squamous cell carcinoma showed 80 per cent immunoreactivity. Simpson *et al.* (2004) demonstrated immunoreactivity in the cytoplasmic component and occasionally in the nuclei of myoepithelial cells. Moreira *et al.* (2005) found that 14-3-3 σ immunoreactivity was restricted to epithelial cells and significantly stronger in the myoepithelial cells that lined the mammary ducts and lobules.

In the malignant mammary tumours, p63 and 14-3-3 σ immunoreexpression revealed a myoepithelial phenotype which was not always identified by routine histopathology (Destexhe *et al.* 1993; Griffery *et al.*, 1993).

VI. CONCLUSION

The present study findings indicate that p63 expression was restricted to nucleus of myoepithelial cells and 14-3-3 σ immunoreactivity was noticed both in the cytoplasmic and nuclear components. To our knowledge, no reports on 14-3-3 σ expression were available in canine mammary tissues and further detailed study on 14-3-3 σ protein expression in canine mammary tissues should be carried out to screen canine mammary tumours and to obtain a better prognosis.

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