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Of humans and canines: A comparative evaluation of heat shock and apoptosis-associated proteins in mammary tumors

Ramasamy Kumaraguruparan ^a, Devarajan Karunakaran ^b, Chidambaram Balachandran ^c,
Baktavachalam Murali Manohar ^c, Siddavaram Nagini ^{a,*}

^a Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar-608 002, Tamil Nadu, India

^b Division of Cancer Biology, Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram-695 014, Kerala, India

^c Department of Veterinary Pathology, Madras Veterinary College, Chennai-600 007, Tamil Nadu, India

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Abstract

Background: Mammary tumors are the most common neoplasms in humans and canines. Human and canine mammary tumors share several important epidemiological, clinicopathological and biochemical features. Dysregulation of normal programmed cell death mechanisms play an important role in the pathogenesis and progression of breast cancer. We investigated the expression of heat shock proteins (Hsps) as well as apoptosis-associated proteins in both human and canine mammary tumors.

Methods: Twenty breast cancer patients who were categorized as pre- and postmenopausal and 20 mammary gland tumors obtained from dogs were included in this study. The expression of Bcl-2, Bcl-X_L, Bax, caspases 8 and 3 as well as Hsp 70 and 90 in tumor tissues and adjacent tissues were investigated using Western blotting.

Results: While expression of Bcl-2, Bcl-X_L, Hsp 70 and 90 was increased, expression of Bax and caspases 8 and 3 was significantly lower in both human as well as canine mammary tumor tissues compared to corresponding adjacent tissues. The magnitude of the changes was however more pronounced in premenopausal patients compared to postmenopausal patients.

Conclusions: The shift of balance towards expression of Hsp and antiapoptotic proteins may lead to evasion of apoptosis both in humans and canines. The similar pattern of changes in Hsps and apoptosis-associated proteins in human and canine mammary tumors validate use of the canine model to understand the molecular mechanisms of mammary carcinogenesis.

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Keywords: Apoptosis; Bcl-2; Breast cancer; Canines; Caspases; Heat shock proteins

1. Introduction

Breast cancer is the most common cancer worldwide with 910,000 new patients being diagnosed annually [1]. In Southern India, breast cancer is the second most common cancer among women [2]. The etiology of breast cancer is multifactorial. Risk factors include early menarche, late menopause, nulliparity, late age at first

childbirth, postmenopausal obesity, extended use of oral contraceptives, hormone replacement therapy, family history and previous benign breast disease. The common risk factor in the development of breast cancer is the increased lifetime exposure to endogenous or exogenous estrogens [3].

Mammary tumors are the most common neoplasms in most mammalian species including canines. Both benign and malignant neoplasms account for approximately 50% of all tumors in dogs. The incidence of mammary carcinomas in canines is 3 times that documented in humans [4]. The major risk factors associated with canine mammary tumors include increased age, intact status or ovariectomy after 2.5 y of age, nulliparity, irregular estrus cycles, cystic hyper-

* Corresponding author. Tel.: +91 4144 239842; fax: +91 4144 238145/238080.

E-mail addresses: s_nagini@yahoo.com, snlab@sanchamnet.in (S. Nagini).

plasia of the endometrium, obesity early in life, diet rich in beef and pork and progestin treatment [5].

Human and canine mammary tumors share several important epidemiological, morphological, clinicopathological and biochemical features. The incidence of both human and canine breast cancer increases with age. In both species, the majority of the malignant breast tumors arises from epithelial tissue and metastasizes to the lungs or other organs [4,6,7]. Recently, we documented changes in the cellular redox status and xenobiotic metabolizing enzymes as well as altered expression of proteins associated with cell proliferation, apoptosis and differentiation in mammary tumors in both humans and canines [8–12].

Development of mammary tumors involves aberrant accumulation of cells caused either by excessive proliferation or insufficient apoptosis or both. Carcinogenesis selects against apoptosis in order to initiate, promote and perpetuate the malignant phenotype [13]. The Bcl-2 family of proteins in association with caspases plays a central role in regulating apoptosis [14]. The heat shock proteins (Hsp), which function as molecular chaperones are known to inhibit apoptosis [15]. Among different inducible Hsps (Hsp 10, –27, –60, –70), Hsp 70 exerts antiapoptotic effect by binding apoptosis protease activating factor-1 (Apaf-1), thereby preventing formation of the apoptosome complex [16,17]. Although apoptosis evasion has been reported in human breast cancer patients, it has not been sufficiently documented in canine mammary tumors. The present study was therefore designed to investigate the expression of the apoptosis-associated proteins Bcl-2, Bcl-X_L, Bax, caspases 8 and 3, as well as Hsp 70 and 90 in both human and canine mammary tumors.

2. Materials and methods

Twenty newly diagnosed patients with breast cancer, ranging in mean age 45.22 ± 8.85 y from Rajah Muthiah

Table 1
General characteristics of breast cancer patients

Total number of subjects	20
Age range (y)	45.22 ± 8.85
Age at menarche	12–15
Menopausal status	
Premenopausal	10
Postmenopausal	10
Cancer site	Left/Right breast
Clinical status	Infiltrative/Intraductal carcinoma
Morphology	Infiltrative
Clinical stage	
Stage I T ₁ N ₀ M ₀	5
Stage II T ₂ N ₁ M ₀	12
Stage III T ₃ N ₁ M ₀	3

T—Tumour size in diameter; T₁ ≤ 2 cm; T₂ 2–4 cm; T₃ ≥ 4 cm.

N—Regional lymph node metastasis; N₀=No regional lymph node metastasis.

N₁—Metastasis in a single ipsilateral regional lymph node of <3 cm diameter.

M—Distant metastasis; M₀=No evidence of distant metastasis.

Table 2
Reproductive and clinical histories of canines

Total number	20
Age range (y)	7.96 ± 2.35
History of ovariectomy	
<2 y	5
>2 y	13
None	2
Age at first estrus (months)	8.0 ± 2.0
Age at first parturition	
<2 y	3
>2 y	11
None	6
Clinical stage	
Stage I T ₂ N ₀ M ₀	2
Stage II T ₂ N ₀ M ₀	3
Stage III T ₃ N ₀ M ₀	4
Stage IV T ₂ N ₁ M ₀	11

T: Primary tumour size in diameter. T₁ = <3 cm T₂ = 3–5 cm T₃ = >5 cm.

N: Regional lymph node metastasis. N₀=no evidence of regional lymph nodes involved, N₁=ipsilateral regional lymph nodes involved, N₂=bilateral regional lymph nodes involved.

M: Distant metastasis. M₀=no evidence of distant metastasis, M₁=distant metastasis including distant nodes.

Medical College and Hospital, Annamalai University, India who had not undergone any previous treatment for their tumors were chosen for the study. They were categorized according to hormonal status as pre- and postmenopausal patients. Of the 20 breast cancer patients, 10 (50%) were premenopausal and 10 (50%) were postmenopausal. The patients were not using hormones, oral contraceptives and were nonsmokers. None had concomitant diseases such as diabetes mellitus, liver diseases and rheumatoid arthritis. Informed consent was obtained from all the participants. The Human Ethics Committee, India approved the study. Table 1 shows the general characteristics of breast cancer patients.

Twenty mammary gland tumors obtained from dogs were included in this study. Animals ranging in age 5–12 years were clinically examined and surgically treated in the Tamil Nadu Veterinary College and Hospital, Chennai, India. Clinical and reproductive histories were obtained from the owners. The variables included age, history of ovariectomy, age at first oestrus, age at first parturition and the number of full-term pregnancies (Table 2).

The tumor tissues were subdivided and variously processed for distribution to each experiment. For histopathological examination, tissues were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The remaining tissues were immediately stored at –70 °C until use.

2.1. SDS-PAGE and Western blot analysis

Approximately, 50 mg of each tissue sample was homogenized in RIPA buffer containing 50 mmol Tris–HCl (pH 7.4), 1% NP 40, 40 mmol/l sodium fluoride, 10 mmol/l NaCl, 10 mmol/l sodium orthovanadate, 1 mmol/l

phenylmethylsulfonyl fluoride and 10 mmol/l dithiothreitol (DTT) and 1 $\mu\text{g/ml}$ each of leupeptin and aprotinin. The homogenates were centrifuged and the protein content of the supernatants determined by the method of Bradford [18]. SDS-PAGE was performed using equivalent protein extracts (50 $\mu\text{g/lane}$) from each sample according to Laemmli [19]. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilion, Millipore, Bedford, MA). The membranes were incubated in TBS-T (50 mmol/l Tris, pH7.4, 150 mmol/l NaCl, 0.05% Tween 20) containing 5% non-fat dry milk to block nonspecific binding sites for 1 h. The blots were incubated with 1:1000 dilution of appropriate antibodies (Santa Cruz Biotechnology, CA) for 1 h at room temperature and extensively washed with TBS containing 0.05% Tween-20. Bcl-2, Bcl-X_L, Bax, caspases 8 and 3 and Hsp 70 and 90 were detected by incubating with corresponding alkaline phosphatase (ALP)—secondary antibodies (1:5000) for 30–45 min at room temperature. The Hsp 70 antibody used in this study was inducible Hsp 70 which does not react with the constitutive protein of the Hsp 70 (Hsc 70) family. The proteins were visualized by nitrobluetetrazolium/5-bromo-4-chloro-3-indolyl phosphate reagent. Densitometry was performed on IISP flat bed scanner and quantitated with Total Lab 1.11 software.

2.2. Colorimetric estimation of caspase 3 activity

DEVD-specific caspase 3 activity was assayed using CASP-3-C colorimetric kit (Sigma, St. Louis MO) according to the manufacturer's instructions. Cytosolic extracts were prepared by homogenizing tissues in lysis buffer containing 50 mmol/l HEPES (pH 7.4), 5 mmol/l CHAPS and 5 mmol/l DTT. The supernatant was collected as an enzyme source. The caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl–Asp–Glu–Val–Asp–nitroanilide (Ac–DEVD–pNA) by caspase 3, resulting in release of the *p*-nitroaniline (pNA) moiety. The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

2.3. Statistical analysis

Statistical analysis for densitometric analysis was carried out using Student's *t* test. The values are expressed as mean \pm SD. The results were considered statistically significant if the $P < 0.05$.

3. Results

Fig. 1 shows the expression of Bax, Bcl-2, Bcl-X_L, caspases 8 and 3 and Hsp 70 and 90 in tumor and adjacent uninjured tissues of both humans and canines. Using

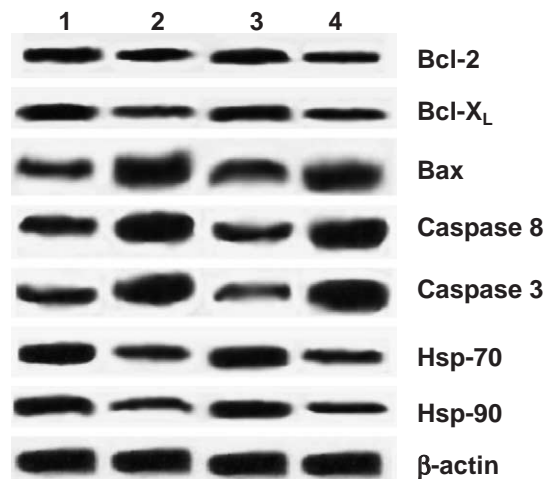


Fig. 1. The expression of Bcl-2, Bcl-X_L, Bax, caspases 8 and 3 and Hsps 70 and 90 in mammary tumor tissues and adjacent tissues of both humans and canines. Protein samples (50 $\mu\text{g/lane}$) resolved on 10% SDS-PAGE were probed with anti-Bcl-2, Bcl-X_L, Bax, Caspases 8 and 3 and Hsp 70 and 90. (1) Human mammary tumor tissue, (2) Human adjacent normal tissue (3) Canine mammary tumor tissue (4) Canine adjacent normal tissue.

immunoblotting, the expression of Bcl-2, Bax, Bcl-X_L, caspases 8 and 3 and Hsp 70 and 90 was detected as bands with molecular weight of approximately 25, 21, 28, 57, 32, 70 and 90 kDa, respectively. The individual and mean values for the apoptosis-associated proteins are presented in Tables 3 and 4. Bcl-2, Bcl-X_L, and Hsp 70 and 90 expression was significantly increased, whereas expression of Bax and caspases 8 and 3 was significantly decreased in tumor tissues compared to adjacent uninjured tissues in both humans and canines. The Bcl-2/Bax ratio showed 6- and 2-fold increase over corresponding adjacent tissues in humans and canines respectively.

Fig. 2 shows the expression of Bcl-2, Bax, Bcl-X_L, caspases 8 and 3 and Hsp 70 and 90 in breast tumor tissues in pre- and postmenopausal patients. Table 5 presents the individual and mean values of protein expression. While expression of Bcl-2, Bcl-X_L and Hsp 70 and 90 was increased, expression of Bax and caspases 8 and 3 was significantly lower in both pre- and postmenopausal breast tumors compared to corresponding adjacent tissues. The Bcl-2/Bax ratio showed 5 and 3 fold increase in pre- and postmenopausal tumor tissues respectively, over their corresponding adjacent tissues. The extent of changes was however greater in premenopausal breast tumors when compared to postmenopausal tumors.

DEVD-specific caspase 3 activity in both human and canine mammary tumors is shown in Fig. 3. The enzyme activity was significantly reduced in tumor tissues compared to adjacent uninjured tissues in humans. A similar pattern was also observed in canine mammary tumors compared to adjacent uninjured tissues. Although the activity of DEVD-specific caspase 3 was significantly reduced in tumor tissues of both pre- and postmenopausal

Table 3
The expression of Bcl2, Bax, Bcl2/Bax ratio and Bcl-X_L in mammary tumor and adjacent normal tissues of humans and canines

S. No	Humans (n=20)								Canines (n=20)							
	Bcl2		Bax		Bcl-2/Bax		Bcl-X _L		Bcl2		Bax		Bcl-2/Bax		Bcl-X _L	
	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N
1	145.07	71.81	63.41	218.04	2.50	0.34	162.27	65.03	134.92	81.64	68.71	178.98	2.16	0.47	158.78	70.11
2	126.47	57.47	53.21	187.04	2.12	0.27	140.43	55.16	119.84	64.90	57.97	155.65	1.82	0.39	137.68	60.13
3	161.19	84.24	72.26	244.90	2.83	0.40	181.20	73.58	147.99	96.15	78.02	199.20	2.45	0.54	177.06	78.76
4	111.59	46.00	45.04	162.24	1.81	0.22	122.96	47.26	107.77	51.51	49.38	136.98	1.55	0.32	120.80	52.14
5	178.55	97.62	81.79	273.83	3.19	0.46	201.58	82.79	162.07	111.77	88.04	220.98	2.77	0.62	196.75	88.08
6	96.71	34.53	36.87	137.44	1.50	0.17	105.49	39.36	95.70	38.12	40.79	118.31	1.28	0.25	103.92	44.15
7	106.63	42.18	42.32	153.97	1.71	0.20	117.14	44.63	103.75	47.05	46.52	130.76	1.46	0.30	115.17	49.48
8	181.03	99.54	83.15	277.96	3.24	0.47	204.50	84.11	164.08	114.01	89.48	224.09	2.81	0.63	199.56	89.41
9	114.07	47.91	46.40	166.37	1.86	0.23	125.87	48.58	109.78	53.74	50.81	140.09	1.60	0.33	123.61	53.47
10	121.51	53.65	50.48	178.77	2.01	0.26	134.61	52.52	115.81	60.44	55.11	149.42	1.73	0.36	132.05	57.46
11	156.23	80.42	69.54	236.63	2.73	0.38	175.38	70.95	143.97	91.69	75.16	192.98	2.36	0.52	171.43	76.10
12	136.39	65.12	58.65	203.57	2.32	0.31	152.08	60.42	127.88	73.83	63.70	168.09	2.00	0.43	148.93	65.45
13	150.03	75.64	66.14	226.30	2.60	0.36	168.10	67.66	138.94	86.11	71.58	185.20	2.25	0.49	164.40	72.77
14	123.99	55.56	51.85	182.91	2.07	0.27	137.52	53.84	117.83	62.67	56.54	152.54	1.78	0.38	134.87	58.80
15	154.99	79.46	68.86	234.57	2.70	0.38	173.92	70.29	142.96	90.57	74.44	191.42	2.34	0.51	170.03	75.43
16	117.79	50.78	48.44	172.57	1.94	0.24	130.24	50.55	112.80	57.09	52.96	144.76	1.66	0.35	127.83	55.47
17	142.59	69.90	62.05	213.90	2.45	0.33	159.36	63.71	132.91	79.41	67.28	175.87	2.11	0.46	155.96	68.78
18	105.39	41.22	41.64	151.91	1.68	0.20	115.68	43.97	102.74	45.93	45.80	129.20	1.44	0.29	113.77	48.81
19	167.39	89.02	75.66	255.23	2.96	0.42	188.48	76.87	153.02	101.73	81.60	206.98	2.56	0.57	184.09	82.09
20	130.19	60.34	55.25	193.24	2.19	0.29	144.80	57.13	122.85	68.25	60.12	160.31	1.89	0.40	141.90	62.12
Mean±SD	136.39±24.90*	65.12±19.20	58.65±13.66*	203.57±41.49	2.32±0.51*	0.31±0.09	152.08±29.23*	60.42±13.21	127.88±20.19*	73.83±22.41	63.70±14.38*	168.09±31.23	2.00±0.45*	0.43±0.11	148.93±28.24*	65.45±13.36

Mean±SD; n=20. Densitometric analysis

T—Tumour tissue; N—Adjacent normal tissue.

*As compared to adjacent normal tissue $P < 0.05$.

*As compared to adjacent normal tissue $P < 0.05$.

Table 4
The expression of caspases 8 and 3 and Hsps 70 and 90 in mammary tumor and adjacent normal tissues of humans and canines

S. No	Humans (n=20)								Canines (n=20)							
	Caspase 8		Caspase 3		Hsp 70		Hsp 90		Caspase 8		Caspase 3		Hsp 70		Hsp 90	
	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N
1	76.93	184.66	71.92	132.45	184.80	77.27	194.99	77.89	68.70	207.00	51.28	149.05	193.80	73.29	185.41	73.84
2	65.55	158.08	62.66	113.46	158.36	65.83	166.10	66.41	60.34	175.87	43.39	129.59	165.10	63.28	158.23	62.27
3	86.80	207.70	79.94	148.91	207.71	87.19	220.03	87.84	75.94	233.97	58.12	165.91	218.68	81.96	208.97	83.86
4	56.44	136.82	55.26	98.27	137.21	56.67	142.99	57.22	53.66	150.97	37.08	114.03	142.14	55.28	136.49	53.02
5	97.43	232.51	88.58	166.63	232.39	97.87	246.99	98.56	83.74	263.02	65.48	184.07	245.47	91.30	234.34	94.65
6	47.33	115.56	47.86	83.08	116.06	47.51	119.88	48.03	46.98	126.07	30.77	98.47	119.18	47.28	114.75	43.77
7	53.40	129.73	52.79	93.21	130.16	53.62	135.29	54.16	51.43	142.67	34.98	108.84	134.49	52.61	129.24	49.94
8	98.94	236.05	89.81	169.17	235.91	99.40	250.85	100.09	84.85	267.17	66.54	186.66	249.30	92.63	237.96	96.20
9	57.96	140.36	56.49	100.80	140.74	58.20	146.84	58.75	54.77	155.12	38.13	116.62	145.97	56.61	140.11	54.56
10	62.51	151.00	60.20	108.40	151.31	62.77	158.40	63.34	58.12	167.57	41.29	124.41	157.45	60.62	150.99	59.19
11	83.76	200.61	77.47	143.85	200.66	84.14	212.33	84.78	73.71	225.67	56.02	160.72	211.03	79.29	201.72	80.78
12	71.62	172.26	67.60	123.59	172.46	71.93	181.51	72.53	64.80	192.47	47.60	139.97	180.41	68.62	172.73	68.44
13	79.97	191.75	74.39	137.52	191.85	80.32	202.70	80.95	70.93	215.30	53.39	154.24	201.46	75.96	192.66	76.92
14	64.03	154.54	61.43	110.93	154.84	64.30	162.25	64.88	59.23	171.72	42.34	127.00	161.28	61.95	154.61	60.73
15	83.01	198.84	76.86	142.58	198.90	83.38	210.40	84.01	73.16	223.60	55.49	159.43	209.11	78.63	199.91	80.01
16	60.24	145.68	58.35	104.60	146.02	60.49	152.62	61.05	56.45	161.35	39.71	120.52	151.71	58.62	145.55	56.88
17	75.42	181.12	70.69	129.92	181.27	75.75	191.14	76.36	67.59	202.85	50.23	146.46	189.98	71.96	181.79	72.30
18	52.65	127.96	52.18	91.94	128.40	52.86	133.36	53.39	50.88	140.60	34.45	107.55	132.57	51.95	127.43	49.17
19	90.60	216.56	83.03	155.24	216.52	91.01	229.66	91.67	78.73	244.35	60.75	172.40	228.25	85.30	218.03	87.72
20	67.83	163.40	64.52	117.26	163.65	68.12	171.88	68.70	62.02	182.10	44.97	133.49	170.84	65.29	163.67	64.59
Mean±SD	71.62±15.24*	172.26±35.58	67.60±12.39*	123.59±25.42	172.46±35.39*	71.93±15.32	181.51±38.67*	72.53±15.37	64.80±11.18*	192.47±41.66	47.60±10.56*	139.97±26.04	180.41±38.42*	68.62±13.39	172.73±36.38*	68.44±15.18

Mean±SD; n=20. Densitometric analysis.

T—Tumour tissue; N—Adjacent normal tissue.

*As compared to adjacent normal tissue $P < 0.05$.

*As compared to adjacent normal tissue $P < 0.05$.

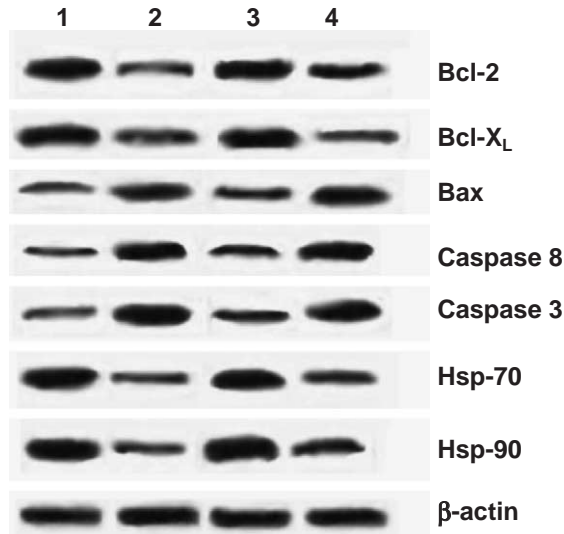


Fig. 2. The expression of Bcl-2, Bcl-X_L, Bax, caspases 8 and 3 and Hsps 70 and 90 in mammary tumor tissues and adjacent tissues of both pre- and postmenopausal patients. Protein samples (50 µg/lane) resolved on 10% SDS-PAGE were probed with anti-Bcl-2, Bcl-X_L, Bax, caspases 8 and 3 and Hsps 70 and 90. (1) Premenopausal tumor tissue, (2) Premenopausal adjacent normal tissue, (3) Postmenopausal tumor tissue, (4) Postmenopausal adjacent normal tissue.

breast tumors, the extent of decrease was greater in premenopausal patients compared to postmenopausal tumors (Fig. 4).

4. Discussion

The human and canine mammary tumors analysed in the present study were characterized by enhanced expression of Bcl-2, Bcl-X_L, and Hsp 70 and 90 with downregulation of Bax and caspases. Bcl-2 and Bcl-X_L are prominent antiapoptotic proteins that inhibit the release of proapoptotic molecules from the mitochondria by multiple mechanisms including maintenance of mitochondrial membrane integrity and binding to proapoptotic members of the Bcl-2 family [14]. While Bcl-2 mediates its antiapoptotic effects by preventing mitochondrial release of cytochrome *c* and consequent activation of caspases, Bcl-X_L forms a ternary complex with Apaf-1 and procaspase 9, preventing caspase 9 activation [20].

Bcl-2 overexpression, a key event in malignant transformation has been reported to be associated with downregulation of Bax [13,21]. Bax, a proapoptotic member of the Bcl-2 family shares extensive amino acid homology with and acts as a functional antagonist to Bcl-2 [22]. Hence, the ratio of Bcl-2/Bax appears to be the best variable in assessing the overall propensity of a cell to undergo apoptosis. Dysregulation of apoptosis due to an imbalance in Bcl-2/Bax ratio has been suggested to contribute to the pathogenesis of breast cancer [21].

Overexpression of antiapoptotic proteins can eventually lead to activation of caspases, a family of intracellular

Table 5
The expression of Hsps and apoptosis associated proteins in mammary tumor and adjacent normal tissues of pre- and postmenopausal patients

	S. No	Bcl2		Bax		Bcl-2/Bax		Bcl-X _L	
		T	N	T	N	T	N	T	N
Premenopausal patients	1	160.70	70.10	49.54	198.58	2.53	0.88	178.72	69.31
	2	149.72	61.65	44.64	180.79	2.32	0.84	162.95	62.83
	3	182.88	87.17	59.45	234.51	2.96	0.97	210.58	82.40
	4	125.98	43.38	34.03	142.31	1.87	0.74	128.84	48.82
	5	165.18	73.55	51.55	205.84	2.62	0.90	185.16	71.95
	6	132.70	48.55	37.03	153.20	2.00	0.77	138.50	52.78
	7	116.59	36.15	29.83	127.10	1.69	0.70	115.36	43.28
	8	180.19	85.10	58.25	230.16	2.91	0.96	206.72	80.81
	9	155.10	65.79	47.04	189.50	2.43	0.86	170.68	66.00
	10	137.18	52.00	39.03	160.46	2.08	0.79	144.93	55.43
Mean ± SD		150.62 ± 22.40* [†]	62.34 ± 17.24	45.04 ± 10.01* [†]	182.24 ± 36.30	2.34 ± 0.43* [†]	0.084 ± 0.09	164.24 ± 32.18* [†]	63.36 ± 13.22
Postmenopausal patients	1	134.84	76.90	79.73	245.76	1.83	0.64	151.71	62.69
	2	121.02	67.10	71.60	223.05	1.68	0.60	138.87	57.02
	3	162.76	96.70	96.16	291.66	2.12	0.73	177.65	74.14
	4	91.13	45.90	54.00	173.90	1.36	0.50	111.10	44.75
	5	140.48	80.90	83.05	255.03	1.89	0.66	156.95	65.00
	6	99.59	51.90	58.98	187.81	1.45	0.53	118.96	48.22
	7	79.31	37.52	47.04	154.48	1.23	0.46	100.12	39.91
	8	159.37	94.30	94.17	286.10	2.09	0.72	174.50	72.75
	9	127.79	71.90	75.58	234.17	1.75	0.62	145.16	59.79
	10	105.23	55.90	62.30	197.08	1.51	0.55	124.20	50.54
Mean ± SD		122.15 ± 28.20*	67.90 ± 20.00	72.26 ± 16.60*	224.90 ± 46.36	1.69 ± 0.30*	0.60 ± 0.09	139.92 ± 26.20*	57.48 ± 11.57

Mean ± SD; n = 20. Densitometric analysis.

T—Tumour tissue; N—Adjacent normal tissue.

*As compared to adjacent normal tissue P < 0.05.

†As compared to adjacent normal tissue P < 0.05.

‡As compared to postmenopausal tumor tissue P < 0.05.

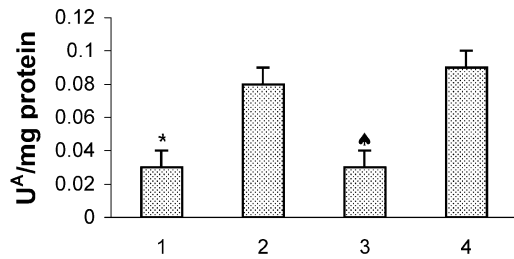


Fig. 3. The activity of caspase 3 in human and canine mammary tumors. (1) Human mammary tumor tissue, (2) Human adjacent normal tissue, (3) Canine mammary tumor tissue, (4) Canine adjacent normal tissue.

proteases that mediate cell death by cleavage of proteins vital for cell survival. Caspase 8, an initiator caspase that links the death receptor and mitochondrial pathways of apoptosis also induces cytochrome *c* release from the mitochondria. Caspase 3 is an effector caspase that cleaves and activates procaspase 8 thereby amplifying and speeding up the apoptotic process [23]. Reports on caspase expression in breast tumors are conflicting. While some studies have reported overexpression of caspases 8 and 3 which may be due to crossreactivity of anticaspase–antibody with some noncaspase proteins in the breast tissue sections [24–26], others including the present one have demonstrated downregulation of caspases in mammary tumors of both humans and canines [23,27,28].

Downregulation of caspases has been demonstrated to be linked to overexpression of Hsps [17,29,30]. Yano et al.

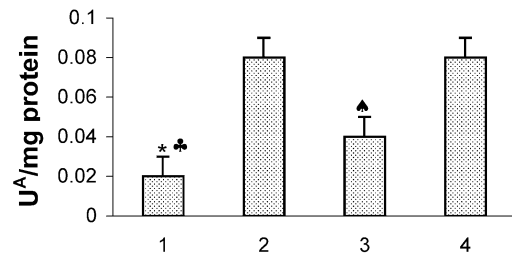


Fig. 4. The activity of DEVD-specific caspase 3 in breast cancer patients with respect to menopausal status, (mean±SD; *n*=10). The activity of DEVD-specific caspase was measured by colorimetric method using Ac–DEVD–pNA as substrate and Ac–DEVD–CHO as inhibitor. * Significantly different from group 2 (*P*<0.05). † Significantly different from group 4 (*P*<0.05). ‡ Significantly different from group 3 (*P*<0.05). A—μmoles of pNA formed/min. (1) Premenopausal tumor tissue, (2) Premenopausal adjacent normal tissue, (3) Postmenopausal tumor tissue, (4) Postmenopausal adjacent normal tissue.

[31] observed a positive correlation between overexpression of Hsp 70 and 90, and high rates of cell proliferation in human breast tumor tissues. Hsps are recognized to induce cytoprotection and rescue cells from apoptosis. Both Hsp 70 and 90 inhibit key steps in the apoptotic cascade such as apoptosome formation, caspase 3 activation and Fas signalling that promote cell survival and contribute to proliferation of a clonal population of malignant cells [15,30,32]. Thus enhanced expression of Bcl-2, Bcl-X_L, and Hsp 70 and 90 associated with downregulation of Bax and caspases seen in human and canine mammary tumors

Caspase 8		Caspase 3		Hsp 70		Hsp 90	
T	N	T	N	T	N	T	N
89.93	77.86	84.04	123.13	207.85	81.42	258.46	78.83
80.89	61.96	77.03	112.20	193.05	72.46	240.57	72.31
108.20	109.97	98.19	145.20	237.75	99.53	294.62	91.99
61.34	27.58	61.87	88.56	161.04	53.07	201.86	58.21
93.62	84.35	86.90	127.59	213.89	85.08	265.77	81.49
66.87	37.31	66.16	95.25	170.10	58.56	212.81	62.20
53.60	13.98	55.88	79.22	148.39	45.41	186.56	52.64
105.98	106.08	96.48	142.53	234.12	97.33	290.24	90.40
85.32	69.75	80.46	117.55	200.30	76.85	249.33	75.50
70.56	43.80	69.02	99.71	176.14	62.22	220.12	64.86
81.63±18.45 ^{*,*}	163.26±32.44	77.60±14.30 ^{*,*}	113.09±22.30	194.26±30.20 ^{*,*}	73.19±18.29	242.03±36.52 ^{*,*}	72.84±13.30
61.12	179.20	57.19	133.97	168.80	76.18	139.22	80.01
79.63	255.25	72.56	175.91	149.05	70.18	119.37	71.53
47.86	124.74	46.19	103.93	208.69	88.30	179.34	97.15
69.75	214.66	64.36	153.52	106.33	57.21	76.42	53.18
51.61	140.16	49.31	112.43	176.86	78.63	147.33	83.47
42.62	103.21	41.84	92.05	118.42	60.88	88.57	58.37
78.13	249.08	71.31	172.51	89.44	52.08	59.44	45.93
64.12	191.54	59.69	140.77	203.86	86.83	174.48	95.07
54.11	150.43	51.38	118.10	158.72	73.12	129.09	75.68
61.12	179.20	57.19	133.97	126.48	63.33	96.68	61.83
61.62±12.51 [*]	181.26±51.38	57.61±10.38 [*]	135.10±28.34	150.66±40.30 [*]	70.67±12.24	120.99±40.52 [*]	72.22±17.31

may facilitate evasion of apoptosis and development of the malignant phenotype.

The shift of balance towards expression of cytoprotective and antiapoptotic proteins was more pronounced in breast tumors in premenopausal than in postmenopausal women. This may be because premenopausal tumors tend to be generally more aggressive than breast tumors that develop in women after menopause. This aspect is especially significant in countries like India, where the incidence of breast tumors is more common in premenopausal women [33]. Evasion of apoptosis, one of the hallmark capabilities of cancer, increases the likelihood of sustaining genetic mutations necessary for malignant transformation by prolonging cell survival. Evasion of apoptosis may also facilitate growth factor- and hormone-independent survival during metastasis, promotes resistance to immune-based destruction and confers resistance to chemotherapy [13,34].

Examination of existing literature has revealed that human and canine mammary tumors have similarities at the molecular level. Mutations in several genes including BRCA1, BRCA2, *c-erbB2* and p53 have been implicated in both human as well as canine mammary tumor development and progression [35–38]. Canine mammary tumors are considered ideal for comparative evaluation of the molecular mechanisms of carcinogenesis than the traditional rodent model [39]. The results of the present study provide further evidence to validate use of the canine model. Furthermore, since pets and their owners share the same environment, comparative studies of diseases in pets may contribute insights into complex human health problems that laboratory experiments cannot provide.

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