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RESEARCH ARTICLE

Evaluation of Bcl-2, Bcl-X_L and Bax Expression and Apoptotic Index in Canine Mammary Tumours [1]

Funda YILDIRIM [1] Kıvılcım SÖNMEZ [1] Hande ÖZYOĞURTÇU [1] Gülbin ŞENNAZLI [1]
Aydın GÜREL [1] Mehmet Can GÜNDÜZ [2] Besim Hasan SONTAŞ [2]

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[1] Istanbul University, Faculty of Veterinary Medicine, Pathology Department, TR-34320 Avcılar, Istanbul - TURKEY
[2] Istanbul University, Faculty of Veterinary Medicine, Gynecology and Obstetrics Department, TR-34320,Avcılar, Istanbul - TURKEY

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Summary

Mammary tumours are the most common neoplasms in intact female dogs. Dysregulation of programmed cell death mechanisms plays an important role in the pathogenesis and progression of mammary gland tumours. The aim of this study was to investigate the relationship between some anti-apoptotic proteins (Bcl-2, Bcl-X_L and Bax), apoptotic index (AI) and histopathological diagnosis, tumour grading, tumour staging and survival time of canine mammary tumours (CMT). Twenty seven tissue samples were collected from twenty seven animals with mammary tumours. The samples were evaluated and graded histopathologically. All cases were staged according to the TNM system. The expression of Bcl-2, Bcl-X_L and Bax proteins was investigated using indirect immunoperoxidase test and apoptosis was evaluated using terminal deoxynucleotidyltransferase (TdT)-mediated nick end-labelling (TUNEL) technique. Follow-up examination and survival estimation analysis were performed. While there was a significant statistical relation between Bcl-2 expression and histopathological diagnosis ($P<0.005$), there was no considerable association between histopathological diagnosis and Bax, Bcl-X_L and AI ($P>0.05$). The differences between T1 and T5, T2 and T5 stages were statistically significant in terms of Bax expression ($P<0.05$), and Bax expressions were higher in T5 when compared with T1 or T2. No association between survival time and Bcl-2, Bax, Bcl-X_L and AI was determined ($P>0.05$). Bcl-2 was overexpressed in highly malignant tumours such as solid and tubulopapillary adenocarcinomas and Bax had high expression levels in metastatic tumours. As a result, it is concluded that Bcl-2 and Bax expression can be accessory parameters for anticipating the biologic behaviour and prognosis of CMT but these markers alone are not sufficient for the determination of survival time.

Keywords: *Canine mammary tumour, Apoptosis, Bcl-2, Bcl-X_L, Bax, Immunohistochemistry, TUNEL*

Köpek Meme Tümörlerinde Bcl-2, Bcl-X_L ve Bax Sunulumu İle Apoptotik İndeksin Değerlendirilmesi

Özet

Meme tümörleri intakt dişi köpeklerin en sık karşılaşılan tümörleridir. Programlı hücre ölüm mekanizmalarında meydana gelen düzensizlikler meme bezi tümörlerinin patogenezis ve progresyonunda önemli bir rol oynamaktadır. Bu çalışmanın amacı köpek meme tümörlerinde (KMT) bazı anti-apoptotik proteinler (Bcl-2, Bcl-X_L and Bax) ve apoptotik indeksin (AI), histopatolojik tanı, tümör derecelendirmesi, tümör evreleri ve kalan yaşam süreleri ile ilişkisini ortaya koymaktır. Meme tümörü olan yirmi yedi hayvandan yirmi yedi biyopsi örneği toplandı. Örnekler histopatolojik olarak incelendi ve derecelendirildi. Bütün olgular TNM sistemine göre evrelendi. Bcl-2, Bcl-X_L ve Bax proteinlerinin sunulumu indirect immunoperoksidaz testi ile ve apoptozis ise terminal deoksinükleotidiltransferaz (TdT)-aracılı nick end-labelling (TUNEL) tekniği ile incelendi. Hasta takibi ve hayatta kalma süresi tahmini analizleri gerçekleştirildi. Bcl-2 sunulumu ve histopatolojik tanı arasında önemli bir istatistiksel ilişki belirlenirken ($P<0.005$), histopatolojik tanı ile Bax, Bcl-X_L ve AI arasında dikkate değer bir ilişki saptanmadı ($P>0.05$). Bax sunulumu açısından T1 ve T5 ile T2 ve T5 evreleri arasında önemli istatistiksel farklılıklar belirlendi ($P<0.05$) ve T1 veya T2 ile karşılaştırıldığında Bax sunulumu T5'te daha yüksekti. Hayatta kalma süresi ile Bcl-2, Bax, Bcl-X_L sunulumları ve AI arasında hiçbir ilişki saptanmadı ($P>0.05$). Bcl-2'nin solid ve tubulopapiller adenokarsinomalar gibi malignitesi yüksek tümörlerde aşırı düzeylerde sunulduğu ve Bax'ın metastazik tümörlerde sunulumunun yüksek olduğu belirlendi. Sonuç olarak, Bcl-2 ve Bax sunulumları KMT'nin biyolojik davranış ve prognozlarını tahmin etmede yardımcı parametreler olabileceği fakat bu belirteçlerin tek başlarına hayatta kalma süresinin belirlenmesi için yeterli olmadığına karar verildi.

Anahtar sözcükler: *Köpek, Meme tümörü, Apoptozis, Bcl-2, Bcl-X_L, Bax, İmmunohistokimya, TUNEL*

İletişim (Correspondence)
+212 4737070/17094
funda@istanbul.edu.tr

INTRODUCTION

Mammary tumours are the most common type of tumours in intact female dogs and they constitute about 50% (40-50% of which are malignant) of all neoplastic cases [1-3]. The pathogenic mechanisms behind the development and progress of canine mammary tumours (CMT) have not been completely clarified yet [4,5]. The evaluation of malignancy potential and prognosis is important for the treatment of CMT. Nevertheless the histopathological diagnosis, which is accepted as the best way for the classification of CMT, sometimes can be insufficient for the definition of prognosis because of tumours' complicated histological types and their different biological behaviours [4,6]. Thereby, in recent years the interest on novel markers such as growth and proliferation factors and apoptosis related genes and proteins is increasing in order to make more reliable diagnosis and to estimate of prognosis and biological behaviours of CMT.

Apoptosis plays a key role in the development of mammary gland. In an healthy mammary gland, cell proliferation and apoptosis are in balance. Besides, there is strong evidence that tumour growth is not only related with uncontrolled cell proliferation but also inhibition of apoptosis [7]. It has been reported that the high rate of apoptosis in human breast cancer is associated with a poor prognosis and more apoptosis is seen in tumours of high grade [8-11]. But, the significance of apoptosis in the evaluation of CMT has not been presented sufficiently yet.

The Bcl-2 family of cytoplasmic proteins plays an important role in the process of apoptosis. Bcl-2 itself is a potent cell survival agent with significant anti-apoptotic activity. Members of Bcl-2 protein family can either promote (Bax, Bid, Bcl-X_S) or supress (Bcl-2, Bcl-X_L) apoptosis in a number of cellular systems [12,13]. In CMT, Yang *et al.*[14] reported that the levels of cell proliferation and apoptosis did not appear to be correlated with the expression of Bcl-2 and Bcl-2 expression were slightly greater in benign CMTs than in their malignant counterparts. There are data stating that while expression of Bcl-2 and Bcl-X_L were increased, expression of Bax was significantly lower in both human breast cancer and CMT tissues compared to corresponding adjacent tissue [15]. Although apoptosis-associated proteins were extensively studied in human breast cancer, there are few studies on CMT. The current study is aimed to evaluate the expression of Bcl-2, Bcl-X_L, Bax, apoptotic index (AI) in different histological types of CMT and statistically investigate their association with tumour grade, tumour stage and survival time.

MATERIAL and METHODS

Twenty-seven cases of CMT routinely submitted to the Dept. of Pathology of the Faculty of Veterinary Med., Istanbul University, were included in the study. Clinical data for investigated dogs are given in *Table 1*. Tumour tissues selected for the study were all located in inguinal lobes. Along with the tumour tissues their associated superficial inguinal lymph nodes were fixed with 10% of neutral buffered formalin, processed by routine methods, and embedded in paraffin. All microscopic evaluations were recorded independently by two pathologists.

Histopathological Evaluation

From paraffin blocks, 5 µm-thick sections were cut and stained by haematoxylin and eosin (H&E) and examined under light microscope. Histologic classification and tumour grading were performed based on the protocol proposed by Goldschmidt *et al.*[16].

Tumour Staging

For staging; tumour's diameter was measured, the presence of tumoural cells in associated lymph nodes was evaluated and animals were checked for distant metastasis by thoracal and abdominal radiography. Canine mammary tumours were staged according to the TNM system, which was recommended by Owen [17]: tumour dimension (T), regional lymph node status (N), distant metastasis (M).

Follow-up

To monitor survival time, the owners of the animals were contacted 2 years later. If an animal was ex, date of its death were recorded. Because the owners of animals did not allow necropsy causes of death could not be established properly. Overall survival was defined as the time from surgery to death due to any cause.

Determination of Fragmented DNA in situ

In histopathological evaluation of tumour material Apoptotic Index (AI) is used as a measure of the extent of apoptosis. Generally it is defined as a percentage of apoptotic cells and bodies in all tumour cell population [18]. To present the apoptotic cells the fracture in DNA were labelled using Terminal deoxynucleotidyltransferase (TdT)-mediated nick end-labelling (TUNEL) technique in paraffin sections, following the procedure of applied kit (Apop Tag® Peroxidase In Situ Apoptosis Detection Kit, EMD Millipore). Sections were treated with 20 µg/ml of Proteinase K (EC 3.4.21.64, Dako Inc.) for 10 min after deparaffinization and rehydration. Later they were treated with 3% H_2O_2 in methanol for 5 min, TUNEL mixture for 1 h (in 37°C) and, subsequently, in Anti-Digoxigenin-Peroxidase for 30 min. Then 2% solution of diamino benzidin (DAB) was applied to the sections and counterstained with methyl green. By microscopic evaluation AI was detected in each section. For this purpose, in each case, TUNEL-positive cells and total cells were counted in 10 random areas, under 40x magnification objective. AI was calculated by the formula: 100x (mean number of TUNEL positive cells in 10 random fields)/(mean number of total cells in 10 random fields) [8].

Table 1. *Histopathological diagnosis and grade, clinical stage, survival (in a 2-year follow-up), Bcl-2, Bax, Bcl-X_L expressions and apoptotic index of 27 malignant canine mammary tumours*

Tablo 1. *Yirmi yedi malign köpek meme tümöründe histopatolojik tanı ve derece, klinik evre, hayatta kalma süresi (2 yıllık takip), Bcl-2, Bax, Bcl-X_L sunulumları ve apoptotik indeks*

Dog no.	Diagnosis	Histological Grade	Clinical Stage				Age	Survival (day)	Bcl-2	Bax	Bcl-X_L	Apoptotic index
			T†	N††	M†††	TNM						
1	Complex Adc	2	2	0	0	2	15	750	+	++	++	1
2	Complex Adc	2	2	0	0	2	10	750	+	+	++	0.4
3	Complex Adc	3	3	1	0	4	11	240	+	++	+	0.1
4	Complex Adc	3	3	1	0	4	13	750	+	++	++	1
5	Complex Adc	2	1	0	0	1	8	750	+	++	+++	2
6	Complex Adc	2	3	0	0	3	8	660	+	++	+	1.5
7	Complex Adc	3	2	0	0	2	13	710	+	+	+	4
8	Complex Adc	3	1	0	0	1	6	750	+	++	+++	0.5
9	Complex Adc	3	1	0	0	1	10	750	++	+	+	11
10	Tubulopapillary Adc	3	1	0	0	1	11	750	++	++	++	1
11	Tubulopapillary Adc	3	2	1	1	5	10	189	+	+++	++	0.4
12	Tubulopapillary Adc	2	1	0	0	1	13	750	+++	+	+++	0.1
13	Tubulopapillary Adc	3	2	1	1	5	12	362	+	++	++	1
14	Tubulopapillary Adc	2	1	0	0	1	10	750	+++	++	++	0.4
15	Tubulopapillary Adc	2	1	0	0	1	11	750	++	++	+++	4
16	Tubulopapillary Adc	2	2	0	0	2	13	750	++	++	++	2
17	Solid Adc	3	3	1	1	5	10	1	+++	+++	+++	1
18	Solid Adc	3	3	1	1	5	12	35	+++	++	+	0.6
19	Solid Adc	3	2	0	0	2	14	370	+++	+++	+++	2
20	Solid Adc	3	1	0	1	5	10	495	+++	+++	++	1
21	Solid Adc	3	3	1	0	4	8	750	+++	+++	+++	0.5
22	Solid Adc	3	2	0	0	2	14	480	+++	+	++	2
23	Solid Adc	3	1	1	1	5	8	170	+++	+++	+++	3
24	Spindle Cell Car	3	3	0	1	5	15	750	+	++	+	0.2
25	Spindle Cell Car	3	1	0	0	1	9	290	+++	+	+++	3
26	Spindle Cell Car	3	3	1	1	5	15	215	++	+++	++	0.5
27	Spindle Cell Car	3	3	1	1	5	12	58	++	++	++	5

† *Tumour diameter,* †† *Reginonal lymhp node metastasis,* ††† *Distant metastasis*

Immunohistochemical Staining

Sections obtained from the tumoural tissues were deparaffinized and rehydrated before treatment with 0.3% H_2O_2 solution in methanol to block the endogenous peroxidase activity at room temperature for 10 min. Then they were subjected to antigen retrieval by incubation with Citrate Buffer solution (10 mM Citric Acid, pH 6.0) in a microwave oven (750 W) for 10 min. The sections were washed three times with phosphate buffered saline (PBS; pH 7.4, 0.1M) and incubated with protein blocking agent (sc-2018, Santa Cruz Biotechnology Inc.) for 10 min to block the nonspecific immunolabelling. Subsequently in a humidity chamber, they were incubated with polyclonal anti-Bcl-2 (sc-492), polyclonal anti-Bcl-X_L (sc-634) and polyclonal anti-Bax (sc-493) antibodies (Santa Cruz Biotechnology Inc.) at a dilution of 1:300 (at room temperature for 90 min). After washing three times with PBS, the slides were treated with secondary antibody kit (sc-2018, Santa Cruz Biotechnology Inc.) containing biotinylated secondary antibody and avidin-peroxidase link (incubated at room temperature in each solution for 25 min). Finally, the sections were treated with 3,3'-diaminobenzidine (DAB) according to manufacturer's protocol (sc-2018, Santa Cruz Biotechnology Inc.), washed three times with PBS, counterstained with Mayer's haematoxylin and coverslipped. For washings steps Tween 20 (0.5 ml/l) was added to the PBS buffer. Intensity of immunolabelling was assessed by examination of 10 representative high-power fields (400x). Positive cells were indicated by brown-coloured cytoplasm. A relative staining intensity based on the proportion of immunolabelled cells was

scored as follows: 0-5%; -, 5-19%; +, 20-59%; ++, ≥60% +++. These scores were regarded as negative, mild, moderate and high respectively [14]. Both in TUNEL and IHC; for positive control, sections from a normal canine thymus were used. For negative control, the primary antibody was substituted with PBS.

Statistics

Kruskal-Wallis analysis was used to determine the significance of tumour types, grades and stages on Bcl-2, Bcl-X_L, Bax expressions and AI. In case significance was found with Kruskal-Wallis analysis, to determine the difference between tumour types, grades and stages, Mann-Whitney U analysis was used. Survival estimates for all animals according to Bcl-2, Bcl-X_L and Bax expressions, AI were analyzed with Kaplan-Meier Test. All data were analyzed with Statistical Package for Social Sciences (SPSS) 13.0 software. The level of significance was set to $P<0.05$.

RESULTS

The mean age of the dogs' at the time of the surgery was 11.15±2.44 (SE=0.47). There was no correlation between age and histopathological diagnosis, tumour grade and stage ($P>0.05$). Radiographically 9 of 27 dogs had pulmonary foci which were accepted as metastasis. From these animals 4 had solid, 3 had spindle cell and 2 had tubulopapillary adenocarcinomas. Considering histopathological diagnosis, CMT were classified into 4 groups: complex (n=9) *(Fig. 1A)*, tubulopapillary (n=7) *(Fig. 1B)*, solid (n=7) *(Fig. 1C)* and spindle cell carcinoma (n=4) *(Fig. 1D)*. Regional lymph node metastases were detected in 10 of 27 cases. All data regarding to animals, histopathological diagnosis, grading, staging, survival time, AI, and apoptotic protein expressions were given in *Table 1*.

When sections were evaluated microscopically, immunopositive labelling was observed in all tumour types with anti-Bcl-2 *(Fig. 2A)*, anti-Bcl-X_L *(Fig. 2B)* and anti-Bax *(Fig. 2C)* antibodies. Bcl-2 expression was most densely in solid carcinomas followed by tubulopapillary and spindle cell carcinomas and was weak in complex carcinomas. The Bax expression was weak in tumours which were labelled strongly with anti-Bcl-2 antibody. Additionally, it was detected that Bax expression was more intensive in tumours with lymph node metastasis. The highest AI were recorded in complex adenocarcinomas *(Fig. 2D)*.

Statistical Findings

The mean value of AI was determined to be 1.82 with ANOVA test. The tumours with AI values lower and equal to this value were accept as "low" and higher then this value were accepted as "high" (AI: low≤1.82<high).

According to Kruskal Wallis Analysis, the statistical relation between histopathological diagnosis and Bcl-2 expression was very significant ($P=0.001$). But, there was no significant association between histopathological diagnosis and Bax ($P=0.082$), Bcl-X_L ($P=0.339$) and AI ($P=0.851$).

With Mann-Whitney U test, all tumour types were compared with each other in terms of Bcl-2 expression. There were statistically significant differences between all tumour types ($P<0.05$), except tubulopapillary and spindle

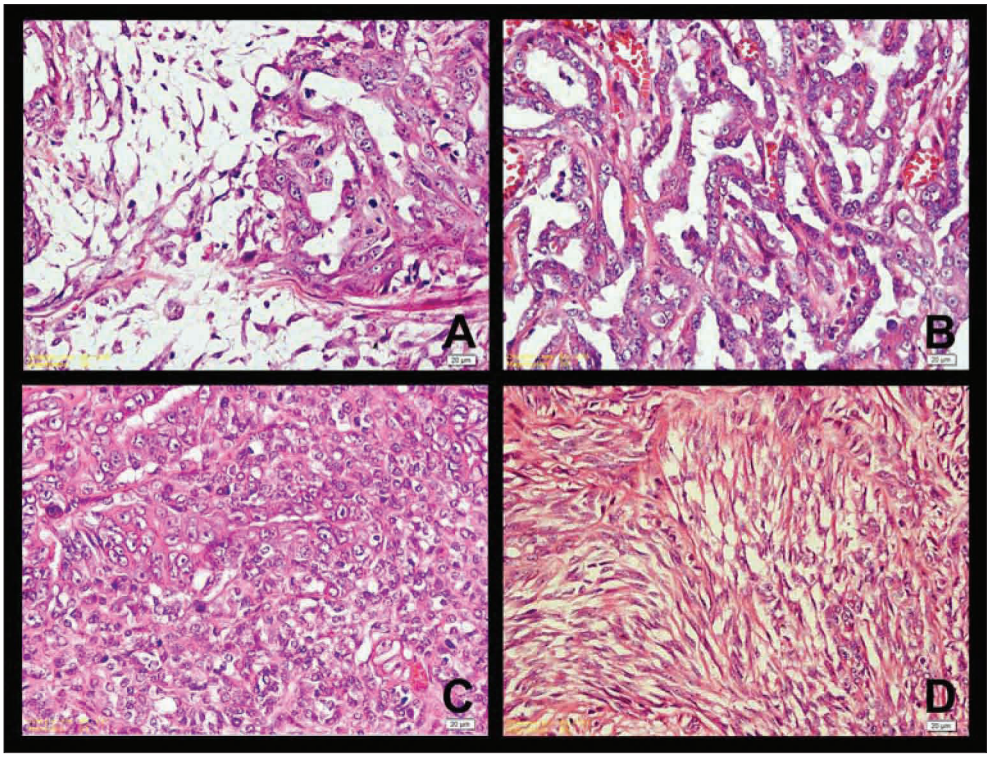


Fig 1. A- Complex adenocarcinoma, Bar=20 µm, H&E; **B**- Tubulopapillary adenocarcinoma Bar=20 µm, H&E; **C**- Solid carcinoma, Bar=20 µm, H&E; **D**-Spindle cell carcinoma, Bar=20 µm, H&E

Şekil 1. A- Kompleks adenokarsinoma, Bar=20 µm, H&E; **B**- Tubulopapiller adenokarsinoma, Bar=20 µm, H&E; **C**- Solid karsinoma, Bar =20 µm, H&E; **D**- Mekik hücreli karsinoma, Bar=20 µm, H&E

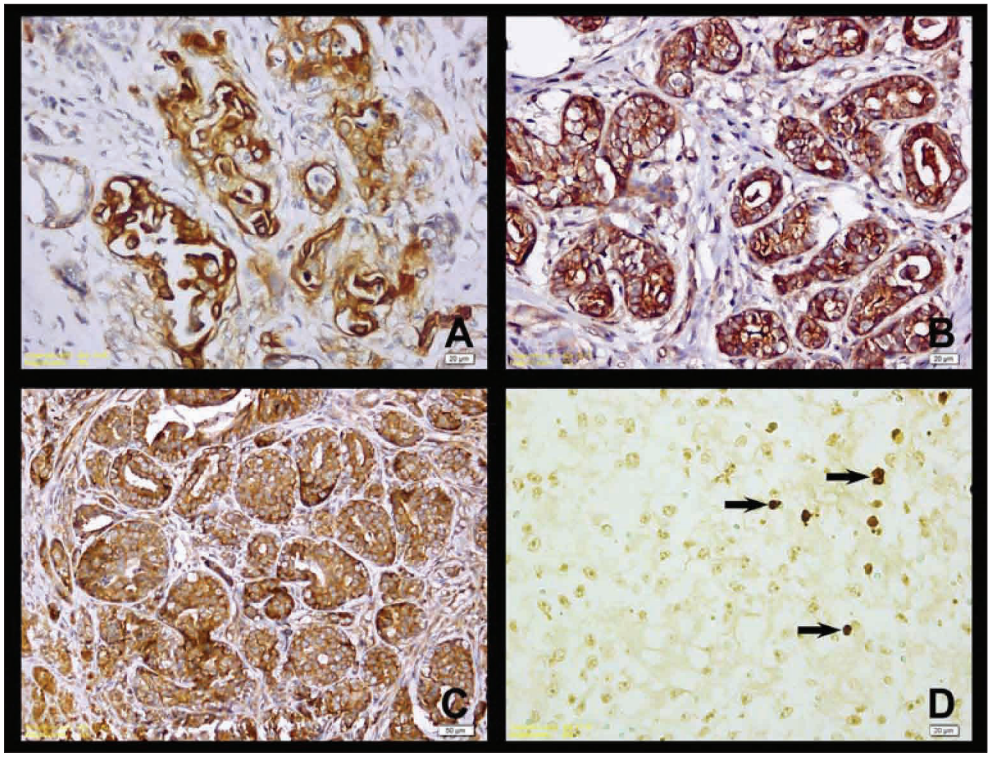
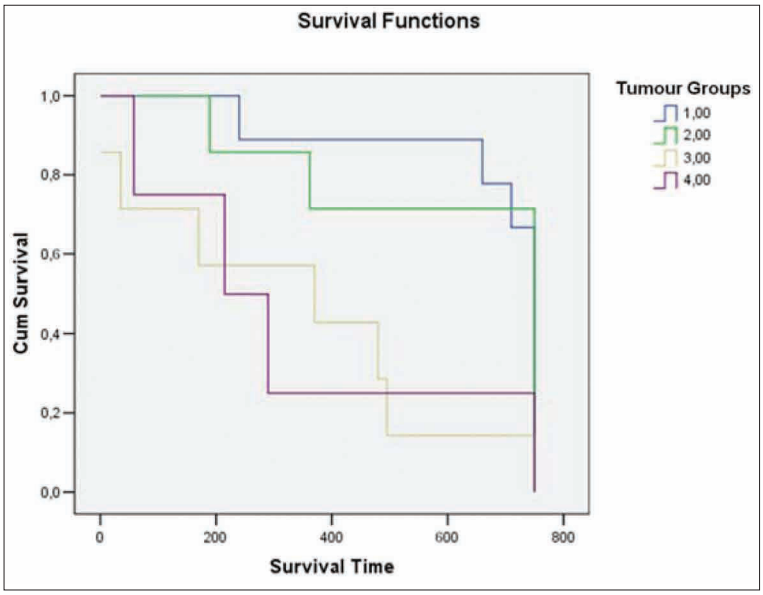


Fig 2. A- Anti-Bcl-2 antibody, high immunopositive reaction (+++), Bar=20 µm; **B-** Anti-Bcl-X_L antibody, high immunopositive reaction (+++), Bar=20 µm; **C-** Anti-Bax antibody, high immunopositive reaction (+++), Bar=50 µm; **D-** TUNEL-positive apoptotic cells (arrows), Bar=20 µm

Şekil 2. A- Anti-Bcl-2 antikoru, kuvvetli immunopozitif reaksiyon (+++), Bar=20 µm; **B-** Anti-Bcl-X_L antikoru, kuvvetli immunopozitif reaksiyon (+++), Bar=20 µm; **C-** Anti-Bax antikoru, kuvvetli immunopozitif reaksiyon (+++), Bar=50 µm; **D-** TUNEL-pozitif apoptotik hücreler (oklar), Bar=20 µm

Fig 3. Kaplan-Meier curve of survival time for dogs with mammary tumours based on histopathological diagnosis (n=27). The first (blue) line indicates dogs with complex adenocarcinomas (n=9, mean survival time 678 days; range, 240-750 days), the second (green) line indicates dogs with tubulopapillary adenocarcinomas (n=7, mean survival time 614 days; range, 189-750 days), the third (yellow) line indicates dogs with solid carcinomas (n=7, mean survival time 328 days; range, 1-750 days) and the forth (purple) line indicates dogs with spindle cell carcinomas (n=4, mean survival time 328 days; range, 58-750 days)

Şekil 3. Meme tümörlü köpeklerin histopatolojik teşhislerine göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) kompleks adenokarsinomalı köpekleri (n=9, ortalama hayatta kalma süresi 678 gün; aralık, 240-750 gün), ikinci çizgi (yeşil) tubulopapiller adenokarsinomalı köpekleri (n=7, ortalama hayatta kalma süresi 614 gün; aralık, 189-750 gün), üçüncü çizgi (sarı) solid karsinomalı köpekleri (n=7, ortalama hayatta kalma süresi 328 gün; aralık, 1-750 gün) ve dördüncü çizgi (mor) mekik hücreli karsinomalı köpekleri (n=4, ortalama hayatta kalma süresi 328 gün; aralık, 58-750 gün) göstermektedir



cell carcinomas (P>0.05).

In Kruskal Wallis Analysis, when the grouping variable was tumour grade, there was no important association between groups. But when the grouping variable was TNM in Kruskal Wallis Analysis, there was significance only with Bax expression (P=0.045). There was statistically significant differences between T1 and T5 (P=0.006), T2 and T5 (P=0.037) in terms of Bax expression through Mann-Whitney U test. There were no other statistically significant differences between other comparisons.

At the follow-up examination, 11 of the dogs were still alive and they were clinically assessed as described. There was no recurrence or distant metastasis in those animals. The shortest survival time periods were in spindle cell carcinoma and solid carcinoma groups *(Fig. 3)*, where 7 of 11 dogs had distant metastasis and higher percentages of Bax expression. The survival curves of histomorphologic groups which were tested daily, significant differences were found between complex and solid adenocarcinomas (P=0.01), complex and spindle cell adenocarcinomas (P=0.02), spindle cell and tubulopapillary adenocarcinomas (P=0.035) by means of Kaplan-Meier Analysis. However, when the survival curves of Bcl-2, Bcl-X_L, Bax and AI were compared, no association was determined between survival time and these parameters (P>0.05) *(Fig. 4)*.

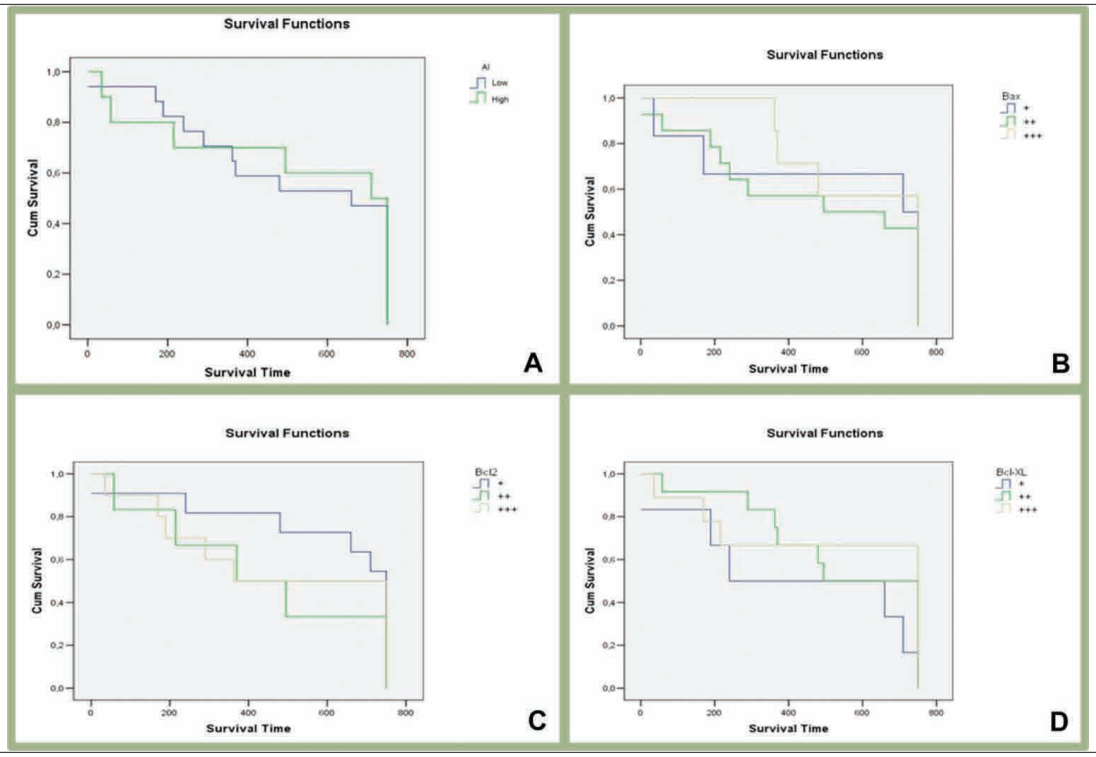


Fig 4. A- Kaplan-Meier curve of survival time for dogs with mammary tumours based on AI (n=27). The first (blue) line indicates dogs with low apoptotic index (n=17, mean survival time 515 days; range, 1-750 days), the second (green) line indicates dogs with high apoptotic index (n=10, mean survival time 526 days; range, 35-750 days); **B-** Kaplan-Meier curve of survival time for dogs with mammary tumours based on Bax expression (n=27). The first (blue) line indicates dogs with mild immunopositive reaction (+) (n=6, mean survival time 527 days; range, 35-750 days), the second (green) line indicates dogs with moderate immunopositive reaction (++) (n=14, mean survival time 474 days; range, 1-750 days), the third (yellow) line indicates dogs with high immunopositive reaction (+++) (n=7, mean survival time 601 days; range, 362-750 days); **C-** Kaplan-Meier curve of survival time for dogs with mammary tumours based on Bcl-2 expression (n=27). The first (blue) line indicates dogs with mild immunopositive reaction (+) (n=11, mean survival time 599 days; range, 1-750 days), the second (green) line indicates dogs with moderate immunopositive reaction (++) (n=6, mean survival time 439 days; range, 58-750 days), the third (yellow) line indicates dogs with high immunopositive reaction (+++) (n=10, mean survival time 479 days; range, 35-750 days); **D-** Kaplan-Meier curve of survival time for dogs with mammary tumours based on Bcl-X_L expression (n=27). The first (blue) line indicates dogs with mild immunopositive reaction (+) (n=6, mean survival time 425 days; range, 1-750 days), the second (green) line indicates dogs with moderate immunopositive reaction (++) (n=19, mean survival time 546 days; range, 58-750 days), the third (yellow) line indicates dogs with high immunopositive reaction (+++) (n=9, mean survival time 546 days; range, 35-750 days)

Şekil 4. A- Meme tümörlü köpeklerin AI'ne göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) düşük apoptotik indeksli köpekleri (n=17, ortalama hayatta kalma süresi 515 gün; aralık, 1-750 gün), ikinci çizgi (yeşil) yüksek apoptotik indeksli köpekleri (n=10, ortalama hayatta kalma süresi 526 gün; aralık, 35-750 gün) göstermektedir; **B-** Meme tümörlü köpeklerin Bax sunulumuna göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) hafif immunopozitif reaksiyonlu köpekleri (+) (n=6, ortalama hayatta kalma süresi 527 gün; aralık, 35-750 gün), ikinci çizgi (yeşil) orta immunopozitif reaksiyonlu köpekleri (++) (n=14, ortalama hayatta kalma süresi 474 gün; aralık, 1-750 gün), üçüncü çizgi (sarı) kuvvetli immunopozitif reaksiyonlu köpekleri (+++) (n=7, ortalama hayatta kalma süresi 601 gün; aralık, 362-750 gün) göstermektedir; **C-** Meme tümörlü köpeklerin Bcl-2 sunulumuna göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) hafif immunopozitif reaksiyonlu köpekleri (+) (n=1, ortalama hayatta kalma süresi 599 gün; aralık, 1-750 gün), ikinci çizgi (yeşil) orta immunopozitif reaksiyonlu köpekleri (++) (n=6, ortalama hayatta kalma süresi 439 gün; aralık, 58-750 gün), üçüncü çizgi (sarı) kuvvetli immunopozitif reaksiyonlu köpekleri (+++) (n=10, ortalama hayatta kalma süresi 479 gün; aralık, 35-750 gün) göstermektedir; **D-** Meme tümörlü köpeklerin Bcl-X_L sunulumuna göre Kaplan-Meier hayatta kalma süreleri eğrileri (n=27). Birinci çizgi (mavi) hafif immunopozitif reaksiyonlu köpekleri (+) (n=6, ortalama hayatta kalma süresi 425 gün; aralık, 1-750 gün), ikinci çizgi (yeşil) orta immunopozitif reaksiyonlu köpekleri (++) (n=19, ortalama hayatta kalma süresi 546 gün; aralık, 58-750 gün), üçüncü çizgi (sarı) kuvvetli immunopozitif reaksiyonlu köpekleri (+++) (n=9, ortalama hayatta kalma süresi 546 gün; aralık, 35-750 gün) göstermektedir

DISCUSSION

The imbalance between cell proliferation and apoptosis is the basis of growth, progression and regression of tumours. Disruption of apoptosis regulation can activate carcinogenesis through different pathways. Apoptosis also limits the tumour cell population at the early stages of tumour development. Decrease in apoptosis may result with cancer by uncontrolled expression of oncogenes or accumulation of malignant cells due to tumour suppressor genes [19,20]. But in some cases the accumulation of neoplastic cells is consistent with increased apoptosis ratio in certain aggressive tumours like human bladder carcinoma [18]. In literature, yet there is little published data on the value of apoptosis as a tool for diagnosis or prognosis in canine tumours. A study by Guvenc *et al.*[21] stated that AI is a useful marker for the differentiation of canine cutaneous histiocytoma and transmissible venereal tumours. Dolka *et al.*[22] reported positive correlation between AI and tumour size, histopathology, grade and proliferative activity. It has

been reported that there is no significant correlation of the survival time with the AI in malignant melanomas of dogs and cats [23]. Funakoshi *et al.*[6] reported that there are no important relationships between AI value of mammary gland tumours and clinical features like metastasis and tumour diameter. In the current study no significant association between AI and histopathological diagnosis, grading and staging of the tumours, survival time were detected. The finding of low degrees of apoptosis in highly malignant tumours or increased apoptosis in some malignant tumours diminished the value of apoptosis as a prognostic tool. So we think that this inconstancy is related to the levels of initiation, development and progression of the cancers.

As Bcl-2 protein is a prosurvival factor which blocks apoptosis one could anticipate that its overexpression would be related with aggressive tumours [24,25]. There are data reporting that overexpression of Bcl-2 is related with down-regulation of Bax and the interruption in the progression of apoptosis is dependent to the imbalance of Bcl-2/Bax, which plays a role in cancer development [15,24,26]. It was reported that in some cancer types such as myeloid leukaemia [27] and cancers of prostate [28], cervix [19] and colon [29] expression of Bcl-2 is increased. But in previous studies with human breast cancers, it was stated that Bcl-2 expression is generally observed in benign proliferative lesions and small, slowly progressing oestrogen-positive, p53-negative tumours and was found to be related with better prognosis [7,25,30]. Yang *et al.*[14] reported that, similarly human breast cancers, Bcl-2 expression is higher in benign CMT than in their malignant counterparts. Contradictory results were reported for Bcl-2 expression in CMT. Kumaraguruparan *et al.*[15,26] stated that they have found higher Bcl-2 expression in tumours than in normal mammary glands. In another study performed in CMT it was reported that no significant correspondence was found between Bcl-2 expression and histology, grade and proliferative activity of CMT [22]. In the present study it was determined that Bcl-2 expression significantly differs between histopathological types of CMT ($P<0.05$). According to the WHO classification; based on differentiation and the biologic behaviour, simple carcinomas can be graded in terms of increasing malignancy as tubulopapillary, solid and anaplastic carcinoma [1] and the simple carcinomas had worse prognosis then complex carcinoma [16]. In the current study appropriately, Bcl-2 expression was highest in solid carcinoma followed by tubulopapillary and spindle cell carcinoma which is a special type mammary gland tumour and was lowest in complex carcinoma. Some researchers reported that by expressing anti-apoptotic proteins like Bcl-2, tumour cells develop a mechanism to avoid apoptotic death signals and gain resistance against apoptosis [31,32]. We concluded that this mechanism can be considered to be the cause of overexpression of Bcl-2 in highly cellular and malignant solid CMT.

In some studies reported in human and canine cancers, the expression of Bax was found to be decreased in malignant tumours when compared to normal tissues and benign tumours [15,29]. On the contrary in the present study Bax expression was higher only in tumours in T5 stage ($P<0.05$). Although there is no sufficient data about the pathogenetic meaning of overexpression of Bax in metastatic tumours, it was reported that proapoptotic and anti-apoptotic gene transcription increases significantly in metastatic tumours [33]. This finding can be explanatory for both Bax and Bcl-2 results obtained.

There are some studies about the decreased expression of proapoptotic Bcl-X_L in canine tumours, but only one article about its relationship with CMT. Kumaraguruparan *et al.*[15] reported that Bcl-X_L expression decreases in CMT compared with normal tissues. In the current study no significant correlation between survival time, different types, grades, stages of CMT and Bcl-X_L expression were determined.

The data obtained from the comparison of survival times and histopathological diagnosis of CMT were compatible with prognostic results of previous literature [1,16]. Some studies on CMT showed that the tumour size, regional lymph node involvement, distant metastasis and histomorphologic characteristics are useful parameters for clinical prognosis [3-5,16]. In the present study, the evaluation of statistical results obtained from comparison between histopathological types, tumour grade and stage showed that the conventional diagnostic methods such as histological grading and TNM staging are reliable factors in estimating the prognosis. This finding supports the previously reported literature above mentioned.

The exact cause of death could not be determined for dogs which died during the study since the owners did not allowed for necropsy. In order to determine the most reliable result for survival time, the disease-free interval or death related to mammary cancer should be determined. Within the bounds of possibilities we could not obtain these data. Therefore we can conclude that to determine the association of survival for the parameters we evaluate, overall survival for any cause of death in dogs was not sufficient. As there was not enough published data about the relation of survival time and Bcl-2, Bcl-X_L, Bax expressions and AI, we couldn't compare with our results adequately.

Most of the studies presenting the relation between CMT, AI and apoptotic proteins are generally about the comparison of normal mammary gland tissue/benign tumours and malignant tumours. In the present study the relation between apoptotic proteins and the histologic types, grade, stage of malignant mammary tumours and survival time were evaluated. Therefore the current study is one of the first studies on this subject. Because only malignant tumours were evaluated in the current study, the diversity between previous literature about apoptotic

proteins and AI can also be explained with this difference.

In conclusion, Bcl-2 was overexpressed in highly malignant tumours such as solid and tubulopapillary adenocarcinomas and Bax had high expression levels in metastatic tumours while they have no association with tumour grade and survival time. Bcl-2 and Bax expression can be accessory parameters for anticipating the biologic behaviour and prognosis of CMT but these markers alone are not sufficient for the determination of survival time. In addition, AI and Bcl-X_L were not sufficient as dependable markers to estimate biologic behaviour and prognosis of CMT with regard to our study's result. Beside the results will contribute to the limited literature about relation between apoptotic proteins and CMT. But it is needed to further investigations with much more sample and further techniques.

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REFERENCES

1. Misdorp W, Else RW, Hellmén E, Lipscomb TP: Histological Classification of Mammary Tumours of the Dog and Cat. AFİP 2nd Series. Vol 7., Washington D.C., 1999.

2. Misdorp W: Tumours of the Mammary Gland. **In,** Meuten DJ (Ed): Tumours in Domestic Animals. 4th ed., 575-606, Iowa State Press, Iowa, 2002.

3. Sorenmo K: Canine mammary gland tumors. *Vet Clin Small Anim,* 33, 573-596, 2003.

4. Hellmén E: The pathogenesis of canine mammary tumours. *Cancer J,* 9, 1-9, 1996.

5. Yamagami T, Kobayashi T, Takahashi K, Sugiyama M: Prognosis for canine malignant mammary tumours based on TNM and histological classification. *J Vet Med Sci,* 58, 1079-1083, 1996.

6. Funakoshi Y, Nakayama H, Uetsuka K, Nishimura R, Sasaki N, Doi K: Cellular proliferative and telomerase activity in canine mammary gland tumours. *Vet Pathol,* 37, 177-183, 2000.

7. Parton M, Dowsett M, Smith I: Studies of apoptosis in breast cancer. *Brit Med J,* 322, 1528-1532, 2001.

8. Lipponen P, Aaltomaa S, Kosma VM, Syrajänen K: Apoptosis in breast cancer as related to histopathological characteristics and prognosis. *Eur J Cancer,* 30A, 2068-2073, 1994.

9. Mustonen M, Raunio H, Pääkkö P, Soini Y: The extent of apoptosis is inversely associated with bcl-2 expression in premalignant and malignant breast lesions. *Histopathology,* 31, 347-354, 1997.

10. Nishimura R, Nagao K, Miyayama H, Matsuda M, Baba K, Matsuoka Y, Yamashita H, Fukuda M, Higuchi A: Apoptosis in breast cancer and its relationship to clinicopathological characteristics and prognosis. *J Surg Oncol,* 71, 226-234, 1999.

11. Zhang GJ, Kimijima I, Abe R, Watanabe T, Kanno M, Hara K, Tsuchiya A: Apoptotic index correlates to bcl-2 and p53 protein expression, histological grade and prognosis in invasive breast cancers. *Anticancer Res,* 18, 1989-1998, 1998.

12. Hockenberry D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature,* 348, 334-336, 1990.

13. Oltvai ZN, Miliman CL, Korsmeyer SJ: Bcl-2 heterodimerizes *in vivo* with a concerved homolog, Bax, that accelerates programmed cell death. *Cell,* 74, 609-619, 1993.

14. Yang WY, Liu CH, Chang CJ, Lee CC, Chang KJ, Lin CT: Proliferative activity, apoptosis and expression of oestrogen receptor and Bcl-2 oncoprotein in canine mammary gland tumours. *J Comp Pathol,* 134, 70-79, 2006.

15. Kumaraguruparan R, Karunagaran D, Balachandran C, Manohar BM, Nagini S: Of humans and canines: A comparative evaluation of heat shock and apoptosis-associated proteins in mammary tumours. *Clin Chim Acta,* 365, 168-176, 2006.

16. Goldschmidt M, Peña L, Rasotto R, Zappuli V: Classification and grading of canine mammary tumours. *Vet Pathol,* 48, 117-131, 2011.

17. Angélica CB, Estrela-Lima, A: Consensus for the diagnosis, prognosis and treatment of canine mammary tumors. *Braz J Vet Pathol,* 4, 153-180, 2011.

18. Soini Y, Paakkö P, Letho VP: Histopathological evaluation of apoptosis in cancer. *Am J Pathol,* 153, 1041-1053, 1998.

19. Jain D, Sirinvasaon R, Patel FD, Kuman J: Evaluation of p53 and Bcl-2 expression as prognostic markers in invasive cervical cancer stage I1b/III patients treated by radiotherapy. *Gynecol Oncol,* 88, 22-28, 2003.

20. Mukaratirwa S: Prognostic and predictive markers in canine tumours: Rationale and relevance. *Vet Quart,* 27, 52-64, 2005.

21. Guvenc T, Haligur M, Orman NM, Haziroglu R: Mitosis and apoptosis in canine cutaneous histiocytoma and transmissible venereal tumour. *Acta Vet Hung,* 50, 315-321, 2002.

22. Dolka I, Motyl T, Malicka E, Sapierinzyski R: Detection of apoptotic cells and ımmunohistochemical study of Bcl-2, bax and Caspase-3 in canine mammary tumours. *J Comp Pathol,* 143, 315, 2010.

23. Roels S, Tilmant K, Ducatelle R: PCNA and Ki-67 proliferation markers .as criteria for prediction of clinical behaviour of melanocytic tumours in cats and dogs. *J Comp Pathol,* 121, 13-24, 1999.

24. Lowe S, Lin WA: Apoptosis in Cancer. *Carcinogenesis,* 21, 485-495, 2000.

25. Treré D, Montanaro L, Ceccarelli C, Barbieri S, Cavrini G, Santini D, Taffurelli M, Derenzini M: Prognostic relevance of a novel semiquantitative classification of Bcl2 immunohistochemical expression in human infiltrating ductal carcinomas of the breast. *Ann Oncol,* 18, 1004-1014, 2007.

26. Kumaraguruparan R, Prathiba D, Nagini S: Of humans and canines: Immunohistochemical analysis of PCNA, Bcl-2, p53, cytoceratin and ER in mammary tumours. *Res Vet Sci,* 81, 218-224, 2006.

27. Karakas T, Maurer U, Weidmann E, Miething CC, Hoelzer D, Bergmann L: High expression of Bcl-2 mRNA as a determinant of poor prognosis in acute myleoid leucemia. *Ann Oncol,* 9, 159-165, 1998.

28. Quinn DI, Henshall SM, Sutherland RL: Molecular markers of prostate cancer outcome. *Eur J Cancer,* 41, 858-887, 2005.

29. Nehls O, Okech T, Hsieh CJ, Enzenginer T, Sarbia M, Borchard F, Gruenagel H-H, Gaco V, Hass HG, Arkenau HT, Hartmann JT, Porschen R, Gregor M, Klump B: Studies on p53, BAX and Bcl-2 protein Expression and microsatellite instability in stage III (UICC) colon cancer treated by adjuvant chemotherapy: Major prognostic impact of proapoptotic BAX. *Brit J Cancer,* 96, 1409-1418, 2007.

30. Silvestrini R, Veneroni S, Daidone MG, Benini E, Boracchi P, Mezzetti M, Di Fronzo G, Rilke F, Veronesi U: The Bcl-2 protein: a Prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. *JNCI J Natl Cancer Inst,* 86, 499-504, 1994.

31. Bettaieb A, Dubrez-Daloz L, Launay S, Plenchette S, Rebe C, Cathelin S, Solary E: Bcl-2 proteins: Targets and tools for chemosensitisation of tumour cells. *Curr Med Chem Anti-Cancer Agents,* 3, 307-318, 2003.

32. Reed JC: Apoptosis-targeted therapies for cancer. *Cancer Cell,* 3, 17-22, 2003.

33. Klopfleisch R, Lenze D, Hummel M, Gruber AD: Metastatic canine mammary carcinomas can be identified by a gene expression profile that partly overlaps with human breast cancer profiles. *BMC Cancer,* 10, 618, 2010.