Aberrant cytoplasmic accumulation of retinoblastoma protein in basal cells may lead to increased survival in malignant canine mammary tumours

S. Gautam, N.K. Sood, K. Gupta

Guru Angad Dev Veterinary and Animal Sciences University Ludhiana, Ludhiana, Punjab, India

ABSTRACT: The retinoblastoma susceptibility gene *RB-1* is a tumour suppressor gene that encodes a protein (Rb) that regulates the transition from the G1 phase to the S phase of the cell cycle. Inactivation of the Rb gene has been shown in a variety of human tumours, including breast, ovarian, hepatic, prostatic, and endometrial carcinomas. Although Rb protein is normally expressed in the nuclei of healthy cells, during carcinogenesis there is a partial or complete loss of nuclear expression. Recently, some reports have indicated aberrant cytoplasmic expression of Rb protein. However, little is known about its cytoplasmic expression and significance as a prognostic marker in canine mammary tumours (CMT). The present study was performed on 36 malignant CMT cases in order to assess the mutational status and prognostic significance of Rb in primary malignant CMT. We report an almost complete loss of nuclear expression of Rb protein with corresponding gain of aberrant cytoplasmic expression in basal/myoepithelial cells in CMT. Strikingly, our analysis reveals a significant positive correlation between survival time and cytoplasmic expression of Rb protein in basal cells. Moreover, cytoplasmic expression of Rb protein in basal cells was also correlated with tumour grade and stage.

Keywords: canine mammary tumour; dogs; immunohistochemistry; retinoblastoma protein

List of abbreviations

CMT = canine mammary tumour, ***RB*** = retinoblastoma gene, **Rb** = retinoblastoma protein

Breast cancer is the most frequent malignant tumour in human females, and despite remarkable progress in the field of early diagnosis and adjuvant therapy, morbidity and mortality due to this type of cancer continue to increase (Raica et al. 2009). Its counterpart in canines, i.e. canine mammary tumour (CMT) is the most common neoplasm in bitches (Moe 2001; Sorenmo 2003 and Egenvall et al. 2005) and nearly 41% to 53% of such neoplasms are malignant (Misdorp 2002).

The mammary epithelium is composed of several cell lineages, including luminal, alveolar and basal/myoepithelial cells. Intact basal/myoepithelial cells are an important determinant of normal breast differentiation, and loss of their function could contribute to induction and/or progression of epithelial cancer (Zou et al. 1994; Radice et al. 1997; Slade et al. 1999). The results of Gudjonsson et al. 2002 also underlined the importance of basal/myoepithelial cells in maintaining the polarity of normal breast and their role as structural tumour suppressors. In contrast to breast cancer in humans, myoepithelial/basal cell proliferation is a feature of CMT (Moulton 1990). Immunolocalisation of various histological and tumour markers including cytokeratins, oestrogen receptor, p63 protein, vimentin and α-smooth muscle actin have been assessed in basal cells in cases of CMT (Gama et al. 2003). However, immunolocalisation of retinoblastoma protein (Rb) has until now not been determined in cases of CMT.

CMT are heterogeneous in their pathological features and clinical behaviour (Nieto et al. 2000). Mammary tumours have been postulated to occur due to complex interactions between intrinsic and extrinsic pathways (Koturbash et al. 2011). One of the most commonly involved intrinsic pathways is the down regulation of tumour suppressor genes. Retinoblastoma (RB) is one of the most frequently mutated tumour suppressor genes and is associated with several malignancies in humans, including breast cancer (Cotran et al. 1999). There is also

growing evidence suggesting that loss of *RB* gene function is a key factor in the initiation and/or progression of a diverse group of human cancers, including those of the lung, breast, bladder and prostate (Harbour et al. 1988; Tang et al.. 1988; Yokota et al. 1988). Normally, Rb protein is expressed in the nuclei of healthy cells. However, in cancer, there is a partial or complete loss of such expression (Karimi et al. 2002). For example, expression of the mutant Rb protein has been recorded in the cytoplasm of prostate cancer cells (Claudio et al. 2002). However, little is known about the cytoplasmic expression of Rb in CMT and its significance as a prognostic marker. In the present report immunolocalisation of Rb protein was studied in 36 cases in order to assess its mutational status and prognostic significance in primary malignant CMT.

MATERIAL AND METHODS

Collection of samples. The study was conducted on cases of CMT presented to the Small Animal Clinics of the Department of Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India, between January 2012 and March 2013. In all, samples from 36 cases were collected immediately after tumour resection and fixed in 10% neutral buffered formalin. Formalin-fixed paraffin-embedded tissue sections were stained for detection of Rb protein using immunohistochemistry.

Histological classification, grading and staging of CMT. The tumours were classified on the basis of the latest WHO classification of mammary tumours of dogs (Misdorp et al. 1999) and the WHO-AIFP classification of canine mammary tumours (Misdorp 2002). Histological grading of canine mammary carcinoma was made as per Misdorp W 2002. In the present study, the malignant tumours were staged according to the modified WHO staging system for CMT (Rutteman et al. 2001) employing the TNM system: tumour, lymph node and metastasis.

Immunohistochemistry. For immunohistochemical studies, 4–5 µm thick paraffin tissue sections were cut and mounted on Superfrost positively charged microscopic slides (Fisher Scientific, USA). The slides were later placed in a hot air oven at 60 °C for one hour to melt the paraffin, allowing proper spread of sections**.** Dewaxing and rehydration was performed by dipping tissue sections in EZ-AR™ Common Solution (BioGenex Laboratories Inc., San Ramon, California, USA), and heating at 70 °C for 10 min in the EZ-Retriever™ System (BioGenex Laboratories Inc., San Ramon, California, USA). Antigen retrieval was achieved by HIER at 95 °C for 10 min followed by 98 °C for 5 min. Following HIER, the sections were allowed to cool to room temperature and were washed three times in TRIS Buffered Saline (TBS, pH 7.4–7.6) for 3 min each. Endogenous peroxidase activity was quenched by dipping the tissue sections in 3% H_2O_2 in methanol for 15 min at room temperature in a humidified chamber, followed by three washes with TBS for 3 min each. The sections were then encircled with a hydrophobic pen (BioGenex Laboratories Inc., San Ramon, California, USA). The activity of non-specific proteins was blocked by incubating the sections in ready-to-use normal horse serum-2.5% (Vector Laboratories, Burlingame CA USA) for 15 min at room temperature in a humidified chamber. Thereafter, the sections were incubated with diluted primary antibody (1 : 100) for one hour at room temperature in a humidified chamber. Then, the sections were washed three times with TBS for 3 min each, followed by incubation in immPRESS™ reagent kit peroxidase universal anti-mouse/rabbit Ig (Vector Laboratories, Burlingame CA USA) for 30 min at room temperature in a humidified chamber followed by three washes with TBS of 3 min each. As a negative control, sections were incubated with TBS instead of the primary antibody. The antigen-antibody-peroxidase reaction was visualised by using freshly prepared 3,3'-diaminobenzidine (DAB) solution (30 µl of DAB chromogen with 1 ml of DAB buffer (immPACT™ DAB Peroxidase Substrate Kit-Vector Laboratories, Burlingame, CA, USA). Sections were then washed with distilled water for 5 min and counterstained with Gill's haematoxylin (Merck, Germany) for 30 s and washed in running tap water for 5 min. Finally, the slides were dehydrated in ascending grades of alcohol, cleared in xylene, mounted with DPX and examined under a microscope (BX 53F, Olympus Corporation, Japan).

Positive staining for Rb protein was evaluated semi-quantitatively as per Karimi et al. 2002 as follows: 0–10% = negative; 10–30% = 1+; 30–60% = 2+, and 60–100% = 3+. Only nuclear staining of moderate to high intensity was considered as positive.

Statistical analysis. The immunohistochemical scores of Rb protein in the basal cells were correlated with the survival time of female dogs to

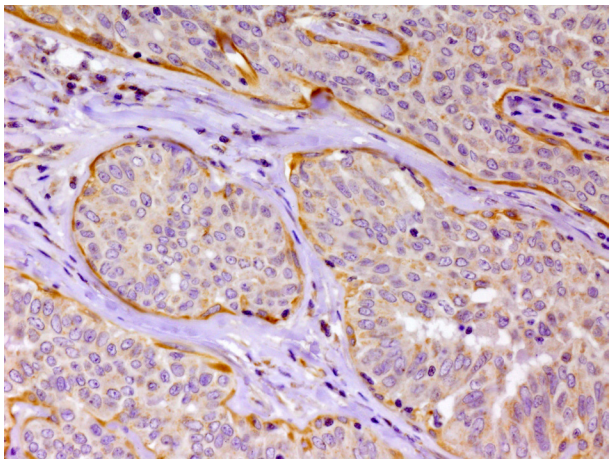

Figure 1. Moderate immunoreactivity of Rb protein in the cytoplasm of basal cells in a solid type simple carcinoma. One step HRP detection system, counterstained with Gill's haematoxylin; original magnification 400×

delineate the significance of Rb protein expression in CMT prognosis using Spearman correlation in SPSS 17.0 software (IBM Corporation). In addition, bivariate correlation analysis was also performed to identify any correlation between Rb expression in basal cells, tumour grade and stage of tumour.

RESULTS

Histological classification of CMT in the present study revealed 19 (52.77%) carcinomas, 14 (38.88%) carcinosarcomas, one (2.77%) sarcoma, and two other tumours. Tumours of grade I, II, and III were present in 31.58%, 31.58%, and 36.84% cases, respectively. Thirty-two out of 36 cases were found to be in advanced stages of malignancy, i.e. stage 4 and 5. Expression of Rb was assessed in all samples using immunohistochemistry. In none of the cases did we observe nuclear staining in more than 10% of cells, indicating a marked loss of expression or an altered expression of Rb protein in all the cases. However, mild to intense cytoplasmic reactivity of

Table 1. Pearson correlation analyses to assess possible associations between survival times of dogs and cytoplasmic expression of Rb protein in cases of CMT

	Survival time (in months)	
Cytoplasmic accumulation of Rb protein in basal cells	pearson correlation	0.417*
	significance (two tailed)	0.022
	n (number of cases)	36

*significant difference at 5% level of significance

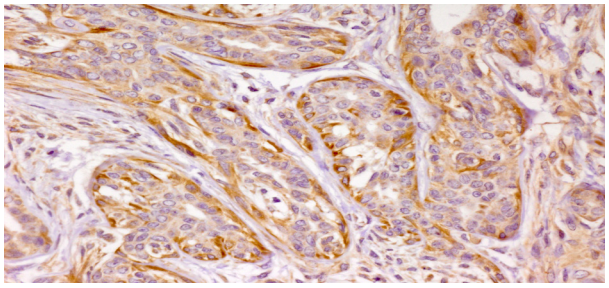

Figure 2. Intense immunoreactivity of Rb protein in the cytoplasm of basal cells and mild cytoplasmic immunoreactivity in epithelial cells. One step HRP detection system, counterstained with Gill's haematoxylin; original magnification 400×

Rb protein was observed in the basal/myoepithelial cells, in most cases (Figure 1 and 2). Mild cytoplasmic reactivity was also observed in epithelial cells. In addition, complete absence of nuclear and cytoplasmic staining was observed in early lesions in different cases of CMT. Mild-to-moderate cytoplasmic reactions were observed in cells invading vascular channels and in tumour emboli (Figure 3).

Spearman correlation analysis between survival time and cytoplasmic expression of Rb protein in the basal cells revealed a positive correlation at 5% level of significance ($P = 0.022$) indicating that the increased localisation of Rb proteins in the cytoplasm of basal cells was associated with better survival in cases of CMT (Table 1). Cytoplasmic expression of Rb protein in basal cells was also correlated with tumour grade and stage. No significant association was found between cytoplasmic immunoreactivity of Rb protein in basal cells and tumour stage ($P = 0.294$) or grade ($P = 0.221$) at 5% level of significance.

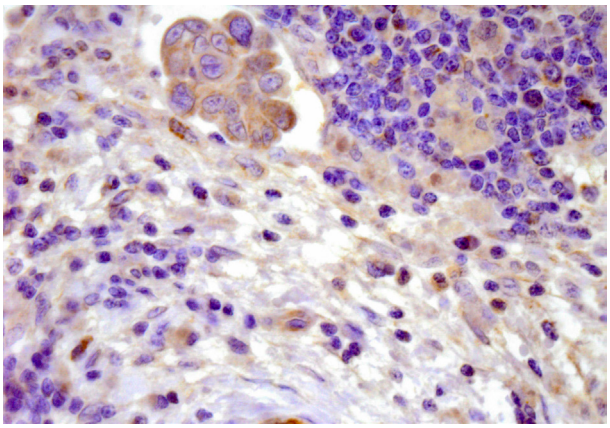

Figure 3. Mild cytoplasmic reactivity in the tumour emboli. One step HRP detection system, counterstained with Gill's haematoxylin; original magnification 1000×

DISCUSSION

It is well known that derangements in the regulation of the natural cell cycle are key phenomena associated with malignant changes. In fact, mutations in at least one of the cell cycle controlling genes, such as Rb protein, are observed in most human malignancies (Cotran et al. 1999). The retinoblastoma susceptibility gene *RB-1* is a tumour suppressor gene that encodes a protein (Rb) that regulates the transition from the G1 phase to the S phase of the cell cycle (Lee et al. 1991; Weinberg 1995). Rb is initially synthesised in the cytoplasm and is then transported into the nucleus, where it exerts its effects. Normally, Rb protein is present in a non-phosphorylated state that binds to and inhibits the transcription factor E2F. Phosphorylation of Rb protein leads to the release of E2F and progression of the cell cycle into S phase.

Inactivation of Rb gene has been shown in a variety of human tumours including breast, ovarian, hepatic, prostate, and endometrial carcinomas (Yeung et al. 1993). Several studies have reported differential levels of Rb inactivation and of involvement of the Rb gene in the pathogenesis and progression of tumours such as lung cancer (Baldi et al. 1997; Helin et al. 1997) and nasopharyngeal carcinoma (Claudio et al. 2000). Although most studies indicate that loss of nuclear expression of Rb protein may be responsible for malignant transformation, no studies have so far attempted to correlate its aberrant cytoplasmic expression with prognosis. Thus, cytoplasmic localisation of Rb protein may be a novel mechanism of inactivation of the Rb protein as reported by Koenig et al. 2002. In the present study, an attempt has been made to determine the localisation of the Rb protein and to delineate its prognostic significance. Mutations in this gene are expected to be characterised by partial or complete absence of nuclear immunoreactivity. Nuclear staining in more than 10% of cells was not observed in any case in the present study. However, mild cytoplasmic reactions in epithelial cells were observed in most cases. Immunoreactivity characterised by intense cytoplasmic staining was also observed in basal cells. Complete absence of immunoreactivity was observed in early lesions.

The cytoplasmic accumulation of Rb protein in basal cells was found to be positively associated with the survival time of dogs with CMT. A prolonged survival time was observed in cases with higher cytoplasmic accumulation in basal cells compared to those with lower levels of cytoplasmic accumulation. Thus, female dogs suffering from CMT with higher expression of Rb in basal cell cytoplasm tended to live longer and had better prognosis. We conclude that loss of nuclear expression of Rb protein and a corresponding gain in aberrant cytoplasm expression particularly in the basal/myoepithelial cells of malignant CMT may serve as an important prognostic determinant.

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Corresponding Author:
Dr. Naresh Kumar Sood, Senior Veterinary Pathologist, Guru Angad Dev Veterinary and Animal Sciences University, Department of Veterinary Pathology, Ludhiana – 141004, India
Tel. (O) +91 161 2414027, (Mo) +91 9888130417, Fax +91 161 2400822, E-mail: nareshsood47@gmail.com