Pathological and immunohistochemical studies on rare cases of primary extra-genital transmissible venereal tumours in the mammary gland

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ABSTRACT: Transmissible venereal tumours (TVT) are normally seen on the genitalia of both male and female dogs, and at times may be observed on extra-genital sites such as lips, oral mucosa, and peritoneum, or in organs such as the tonsils, eye, liver, spleen, kidney, lung, and musculature. The present communication deals with two rare cases of primary extragenital TVT involving the mammary glands of dogs and their pathology and immunohistochemistry. The study indicated that apart from routinely used markers such as vimentin, p53, PCNA, Ki-67 and c-myc, the oncogenes Rb and cyclin D1 proved to be novel markers of TVT in dogs. To the authors' knowledge, this is the first report of extra-genital mammary TVT in canines.

Keywords*:* dogs; extra-genital; immunohistochemistry; mammary gland; primary transmissible venereal tumour

Mammary neoplasms in dogs are the second most common neoplasms after skin tumours (Rezia et al. 2009). Nearly 41% to 53% of the mammary tumours that occur in the bitch are malignant (Misdorp 2002). Canine mammary tumours (CMT) are known for their biological and histomorphologic heterogeneity. The histologic heterogeneity observed in canine mammary carcinoma presents considerable difficulties for classification, and as a result a number of classification systems have been proposed for these tumours (Rezia et al. 2009). On the basis of the latest classification, CMT have been classified into malignant epithelial neoplasms, malignant epithelial neoplasms- special types, malignant mesenchymal neoplasms or sarcomas, malignant mixed mammary neoplasms or cracinosarcomas and benign neoplasms (Goldschmidt et al. 2011). Transmissible venereal tumours (TVT) are transplantable tumours unique to the dog. Owing to the nature of transmission by sexual contact, the external genitalia of either sex are the primary site of these tumours (Albanese et al. 2002). Less commonly, the tumour may also be transmitted to the nasal or oral cavities, skin, and the rectum by sniffing or licking. More rarely, they may be found in other areas, including the lips, oral mucosa, and peritoneum, or in organs such as the tonsils, eye, liver, spleen, kidney, lung, and musculature (Park et al. 2006). However, there are no reports of TVT involving mammary glands. TVT cases are usually vimentin, lysozyme, ACM1 and A-1-antitypsin-positive (Marchal et al. 1997; Mukaratirwa and Gruys 2004), and cytokeratin, S-100, CD3 and p63 negative (Mozos et al. 1996). However, there is little known regarding their immunoreactivity to PCNA, Ki-67, p53, Rb, c-myc and cyclin D1. The present communication deals with two rare cases of primary TVT involving the mammary glands of dogs and their pathology and immunoreaction for intermediate filaments, oncogenes, anti-oncogenes and proliferation markers.

MATERIAL AND METHODS

Collection of samples

The present study was conducted on cases of canine mammary tumours presented to the Small

Animal Clinics of the Department of Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana and the local Private Veterinary Clinics in Ludhiana, India between December 2008 and April 2010. In all, 56 cases of canine mammary tumours were presented, out of which two cases were later found to be TVT. From both cases, samples were collected for cytology, histopathology and immuno-histochemistry.

Cytology, histopathology and Fuelgen reaction

Cytology specimens were collected by fine needle aspiration biopsy (FNAB), smear prepared using the squash technique and were stained according to the Wright-Giemsa method (Jain 1986). Tissue samples after excision biopsy were collected in 10% neutral buffered formalin and embedded in paraffin wax (Leica Microsystem, Paraplast tissue embedding medium, 56 °C) for further processing and 4–5 µ sections were cut. The paraffin sections were stained routinely using the haematoxylin and eosin technique (Luna 1968) and for nuclear Fuelgen reactivity.

Immunohistochemistry

For immunohistochemistry studies 4–5 µ thick paraffin-embedded tissue sections were cut and mounted on Superfrost/Plus, positively charged microscopic slides (Fisher Scientific, USA) and immunohistochemical staining for multi-cytokeratin, vimentin, S-100, CD3, p53, p63, c-myc, retinoblastoma, PCNA and Ki-67 was performed using the advanced SS^{TM} One-Step Polymer-HRP IHC Detection System (BioGenex Laboratories Inc., San Ramon, California, USA). The sections were dewaxed and rehydrated by dipping in EZ-AR^{TM} Common Solution (BioGenex Laboratories Inc., San Ramon, California, USA), and heating at 70 °C for 10 min in EZ-Retriever^{TM} System (BioGenex Laboratories Inc., San Ramon, California, USA) and subsequent antigen retrieval was performed by heat using citrate-based EZ-AR^{TM} 3 Solution 10X in

Table 1. Antibodies used for the immunohistochemical studies

Studie No.	Antibody used	Manufacturer	Clone	Dilution used
1	multi-cytokeratin (5/6/8/18)	Novocastra, Leica Biosystems, Newcastle Ltd., UK	5D3 and LP 34	ready to use
2	anti-vimentin	BioGenex Laboratories Inc., San Ramon, California, USA	V9	ready to use
3	anti-p53 protein	BioGenex Laboratories Inc., San Ramon, California, USA	D07	ready to use
4	anti-p53 protein	Scy Tek Laboratories Logan, Utah, USA	D01	ready to use
5	anti-p63	BioGenex Laboratories Inc., San Ramon, California, USA	4A 4	ready to use
6	anti-proliferating cell nuclear antigen (PCNA)	BioGenex Laboratories Inc., San Ramon, California, USA	PC10	ready to use
7	anti-Ki-67 antigen, proliferating cell	BioGenex Laboratories Inc., San Ramon, California, USA	BGX-297	ready to use
8	S-100	Santa Cruz Biotecnology Inc. California, USA	A-14 (rabbit polyclonal)	1:200
9	anti-c-Myc	Santa Cruz Biotecnology Inc. California, USA	A-14 (rabbit polyclonal)	1:50
10	retinoblastoma tumor suppressor gene product – Rb or p Rb	Santa Cruz Biotecnology Inc. California, USA	C-15 (rabbit polyclonal)	1:50
11	rabbit anti-cyclin D1	BioGenex Laboratories Inc., San Ramon, California, USA	rabbit polyclonal	ready to use
12	anti-CD3	Sigma Aldrich GmbH, Steinheim	rabbit polyclonal	1:200

1 : 10 dilution (BioGenex Laboratories Inc., San Ramon, California, USA). Following antigen retrieval the sections were allowed to cool and brought to room temperature before being washed three times in PBS (pH 7.2–7.4) for 3 min each time. Sections were encircled with a hydrophobic pen. The endogenous peroxidase was quenched with a solution of 3% H_2O_2 in methanol for 15 min at room temperature in a humid chamber, followed by thrice washing with PBS for 3 min each. The sections were then incubated with a ready to use power block (BioGenex Laboratories Inc., San Ramon, California, USA) to block non-specific protein binding for 15 min at room temperature in a humidified chamber. Afterwards, the sections were incubated with primary monoclonal/polyclonal antibodies (Table 1) for 60 min at room temperature in a humidified chamber. The sections were then washed three times in PBS for 3 min each, followed by incubation in Polymer HRP (BioGenex Laboratories Inc., San Ramon, California, USA) for 30 min at room temperature in a humidified chamber and three washes with PBS for 3 min each. The antigen-antibody-peroxidase reaction was developed with a freshly prepared 3,3'-diaminobenzidine (DAB) solution. Sections were later washed in 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 in DPX and examined under a microscope (BX 61, Olympus Corporation, Japan). For each antibody, a negative control was run by replacing the primary antibody with PBS buffer.

Immunohistochemical staining index

The immunohistochemical staining of all the markers was evaluated on the basis of a staining index (Heller et al. 2005) obtained by multiplying the staining distribution and intensity scores. The staining distribution was given a score from 0 to 4, with 0 = 0%, 1 = < 10%, 2 = 10–30%, 3 = 31–60%, and 4 = > 61% of cells staining positive. The staining intensity was defined as the strength of the signal in positive-staining tumours, with – = no signal, + = weak signal, ++ = moderate signal, and +++ = strong signal. The staining index obtained by multiplying the staining distribution and intensity scores gave a range of staining indices of 0–12.

RESULTS

History and clinical examination

The first case was detected in a 12 year-old bitch of undetermined breed. The affected bitch showed a hard, non-ulcerated enlargement (2.5 cm in diameter) on the right sided second pair of the mammary gland. Haematological examination revealed Hb – 14.5 g%, TLC – 12 900, DLC (N – 89%, L – 05% and E – 06%). The second case was recorded in a 3.5 year-old cross bred bitch, which had multinodular tumours (Figure 1) involving the right sided second, third and fourth pairs of the mammary gland. Haematological examination revealed Hb – 20.7 g%, TLC – 12 060, DLC (N – 78%, L – 9% and E – 3%). In both the bitches no growths were observed on any other part of the body including genitalia and no evidence of tumour growths in any vital organ following abdominal ultrasonography and chest radiography was found.

Cytopathology

FNAB smears from both cases revealed monomorphous cells arranged in sheets with occasional individual cells interspersed in places with secretary epithelial cells of the mammary gland with small condensed nuclei and markedly vacuolated pale cytoplasm. The cells had round distinct cytoplasmic borders, with clear or slightly basophilic cytoplasm containing numerous clear small vacuoles arranged along the periphery of the cells. Nuclei were oval to round, having clumped chromatin with one or more prominent nucleoli and frequent and at times abnormal mitoses (Figure 2). Based on cytological findings a tentative diagnosis of TVT was made.

Histopathology

Histopathological examination from both the cases showed neoplastic cells arranged in solid sheets, clusters or cords interlaced by a delicate connective-tissue stroma (Figure 3).The cells were round, with abundant cytoplasm that was either clear or finely granular and had a large nuclear/cytoplasmic ratio. Nuclei showed chromatin clumping and contained one or more prominent nucleoli. A mitotic index of 4.8 in the first case, and 8.5 in the second case was observed. In addition,

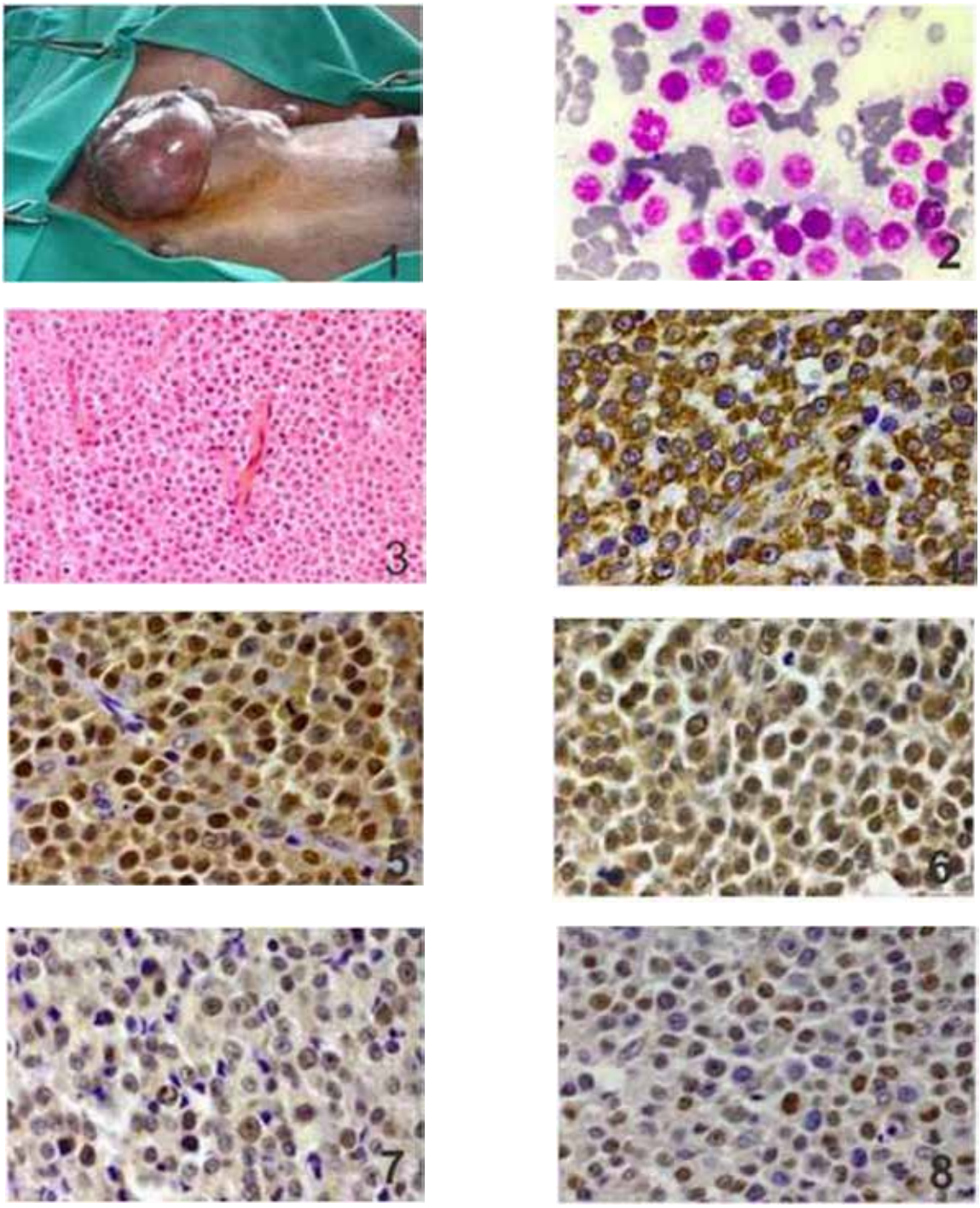

Figure 1–8. **1**. Multi-nodular tumourous growth of the last pair of mammary gland; **2**. Typical TVT cells with abundant vacuolated pale-blue cytoplasm and prominent eccentric hyperchromatic nuclei containing coarse chromatin and a single prominent nucleolus along with a mitotic figure, Wright-Giemsa. Original magnification 1000×; **3**. Typical TVT cells, more or less uniform round to oval in shape, arranged in a cord like fashion along with a scattering of irregular connective tissue strands. H&E. Original magnification 400×; **4**. Moderate cytoplasmic immunoreactivity of vimentin in almost all neoplastic cells; **5**. Strong nuclear PCNA positivity in almost all neoplastic cells; **6**. Moderate cytoplasmic and weak nuclear reactivity of c-myc in the majority of the cells; **7**. Weak to moderate immunoreactivity of Rb in the nucleus as well as cytoplasm; **8**. Moderate to strong nuclear immunoreactivity of cyclin D1 in about half of the neoplastic cells. Note for Fugure 4–8: One step polymer HRP detection system, counter stained by Gill's haematoxylin. Original magnification 1000×

occasional apoptosis of neoplastic cells and moderate Feulgen reaction in the first case, and moderate to strong Feulgen reaction in the second case were also noted. Islands of normal non-lactating acini of mammary glands were detected at the periphery of the tumours.

Immunohistochemistry

Immunohistochemical staining revealed strong cytoplasmic reactivity to vimentin in about 60% cells in the first case whereas this reactivity was moderate and observed in the majority of the cells in the second case (Figure 4). Two clones of p53 were used to assess the immunohistochemical expression of p53. In the first case, weak nuclear to cytoplasmic immunoreaction of Clone 01 of p53 was detected in 30–40% cells invading the connective tissue, while no immunoreaction to Clone 07 of p53 was observed. Conversely, in the second case, weak cytoplasmic reactivity of Clone 07 and no reactivity of Clone 01 of p53 were detected. Moderate nuclear immunoreactivity of PCNA in 60% of the cells was detected in the first case, whereas, in the second case, strong immnunoreactivity of PCNA was detected in about 80–90% cells (Figure 5). No immunoreactivity of Ki-67 was observed in the first case, but strong nuclear immunoreactivity of Ki-67 in about 20–30% cells was observed in the second case. The Feulgen reaction was compatible with PCNA immunoreactivity in both the cases. Weak immunostaining of c-myc in the nucleus as well as cytoplasm in about 50% of cells in the first case and a moderate reaction of c-myc in almost all the cells (Figure 6) in the second case was detected. Moderate immunoreactivity of Rb in the nucleus as well as cytoplasm was observed in about 30% of cells (Figure 7) in the first case, whereas weak immunoreactivity in the majority of cells was observed in the second case. No immunoreactivity of cyclin D1 was observed in the first case; on the other hand, moderate imunoreactivity of cyclin D1 in about 50% cells (Figure 8) was observed in the second case. No immunoreactivity of multi-cytokeratin, S-100, p63 and CD3 was detected in both the cases. The panel of 12 antibodies used in the present study and their respective immunohistochemical indices in both the cases are presented in Table 2.

DISCUSSION

Transmissible venereal tumours mainly affect the external genitalia and occasionally the internal genitalia of dogs. Due to the unique nature of transmission by sexual contact, the external genitalia of

Table 2. Immunohistochemical indices of different markers of Mammary TVT. The staining index obtained by multiplying the staining distribution and intensity scores gave a range of staining indices of 0–12. The staining distribution was given a score from 0 to 4, with 0 = 0%, 1 = < 10%, 2 = 10–30%, 3 = 31–60%, and 4 = > 61% of cells staining positive. The staining intensity was based on strength of the signal in positive-staining tumors, with – = no signal, + = weak signal, ++ = moderate signal, and +++ = strong signal

Studie No.	Antibody used	Imunohistochemical index	
		case No. 1	case No. 2
1	Multi-cytokeratin (5/6/8/18)	0	0
2	anti-vimentin	12	8
3	anti-p53 protein (01 clone)	3 in the cells invading the connective tissue	0
4	anti-p53 protein (07 clone)	0	4
5	anti-p63	0	0
6	anti-proliferating cell nuclear antigen (PCNA)	8	12
7	anti-Ki-67 antigen, proliferating cell	0	6
8	S-100	0	0
9	anti-c-Myc	3	8
10.	retinoblastoma tumor suppressor gene product – Rb or p Rb	6	4
11	cyclin D1	0	6
12	anti-CD3	0	0

either sex are most commonly affected. Less commonly, the tumour may also be transmitted to the nasal or oral cavities, skin, and the rectum by sniffing or licking (Mukaratirwa and Gruys 2004; Park et al. 2006). More rarely, they may be found in other areas, including the lips, oral mucosa, and peritoneum, or in organs such as the tonsils, eye, liver, spleen, kidney, lung, and musculature (Oduye et al. 1973; Park et al. 2006). Recently, Temitope et al (2010) reported metastasis of TVT in the mammary glands of dogs following chemotherapy. However, there are no reports available in the literature on primary TVT involving the mammary glands of dogs and this seems to be the first report of its kind.

The cytolopathological and histoplogical features in both the cases in the present study were similar to those described earlier in genital and extragenital TVT in dogs (Boscos et al. 1998; Das and Das 2000; Mukaratirwa and Gruys 2004; Park et al. 2006; Bastan et al. 2008; Temitope et al. 2010).

Immunohistochemical staining was performed to further confirm TVT and to differentiate it from other round cell tumours. Immunohistochemical staining was positive for vimentin, p53, PCNA, Ki-67, c-myc, Rb and cyclin D1 and negative for cytokeratin, S-100, p63 and CD3. In both the cases, positive reactions for vimentin and a negative reaction for CD3 was observed, which differentiates TVT from lymphomas as documented in earlier studies. The negative reaction for cytokeratin in both cases rules out the possibility of undifferentiated carcinoma, while the negativity for S-100 further rules out amelanotic melanomas, which often express S-100 (Mozos et al. 1996; Ferreira et al. 2000; Park et al. 2006).

TP53 is one of the most important tumour suppressor genes involved in the development of neoplasia. It encodes a nuclear protein, p53 which binds to specific DNA sequences and act as a transcription factor. p53 maintains genomic integrity and controls cell growth. The cellular expression and activity of p53 are closely related. Additionally, p53 has a short half-life (20 min) and is therefore present at low levels within cells (Stockmann et al. 2011). This might be the reason for the weak immunoreactivity of both the clones of p53 used in the present study. Moro et al. (2010) have similarly reported weak immunoreactivity to p53 in natural cases of TVT in dogs. Another reason for the low immunoreactivity of p53 in the present study may be that some anti-human p53 antibodies do not react adequately with canine tissues. So a combination of p53 monoclonal antibodies should be used to screen, not only canine tumours but also human tumours (Haga et al. 2001; Lee et al. 2004). The activation of this protein occurs in response to stress or agents that damage cellular DNA, causing cell cycle arrest and the induction of senescence or apoptosis.TP53 mutations have also been observed in cases of TVT (Sanchez et al. 2009; Stockmann et al. 2011).

Proliferating cell nuclear antigen (PCNA) is an acid nuclear protein which works as a DNA polymerase delta co-factor and is also associated with DNA replication and repair. It is present in all of the phases of the cell cycle, but its synthesis is greater in the S phase. Ki-67 is a non-histone protein with a mean life of less than one hour, which is not expressed in G_0 cells, but can be detected in the active phases of the cell cycle, G_1, S, G_2 and mitosis (Pena et al. 1998; Zuccari et al. 2008). PCNA expression is more intense than that of Ki-67 probably because Ki-67 is restricted to the cell cycle, whereas, PCNA is also expressed in non-cycling cells in association with DNA repair. PCNA also has a long half-life and persists after the end of mitosis (Pena et al. 1998). Both these markers are used for assessing the proliferation index in human as well as animal neoplasms. A strong nuclear reactivity of PCNA was detected in both the cases of TVT, while strong reactivity of Ki-67 was detected in case no. 2 in the present study. However, the detection Ki-67 has proven superior to PCNA in evaluation of the proliferation index in both human and animals tumours (Fournel 1997; Ruiz et al. 2005). Nuclear and cytoplasmic staining of PCNA, and nuclear staining of Ki-67 has similarly been reported earlier in cases of TVT in dogs (Ruiz et al. 2005).

The retinoblastoma (*Rb*) gene encodes nuclear phosphoproteins that regulate cell cycle progression. When Rb protein is in its nonphosphorylated form it inhibits entry of the cell into the S phase of the cell cycle. It does this by binding a transcription factor that stimulates the release of sequestered transcription factors that enables cells to enter the S phase. Following the S phase, the Rb protein is dephosphorylated and is, once again, able to bind transcription factors and inhibit entry into the S phase. In tumour cells, the ability of Rb to bind transcription factors is disrupted and the checkpoint is eliminated. In retinoblastoma, small cell lung carcinomas, and in many sarcomas and bladder carcinomas, pRB function is lost through mutations of the pRB gene (Horowitz et al. 1990). Human cancers frequently contain mutations that

inactivate the Rb pathway either by decreasing the inhibitory activity of Rb and p16 or by deregulating the activity of cyclin D or cdk or by deregulating the activity of cyclin D or cdk. Oncogenic DNA viruses can disrupt cell cycle control by synthesising viral proteins that block the uptake of transcription factors by Rb protein. To date, no reports have appeared regarding the immunohistochemical detection of Rb protein in cases of TVT.

Cyclin D1 is a G_1-specific protein essential for the progression from G_1 phase to S phase; its expression and activity reach a peak in G_1 and gradually decline in S phase (Barbieri et al. 1997). Cyclin D1 plays a pivotal role in the regulation of progression from the G_1 to the S phase of the cell cycle through the formation of active enzyme complexes with cyclin-dependent kinases Cdk4 and Cdk6. These kinases phosphorylate substrates including the retinoblastoma gene product, pRb, thus relieving pRb's inhibitory function on S phase entry. This rate-limiting step in cell cycle progression is regulated by a number of mechanisms including cyclin D1 abundance; consequently, dysregulation of cyclin D1 gene expression or function is a probable contributor to loss of normal cell cycle control during carcinogenesis. Cyclin D1 is one of the most commonly overexpressed oncogenes in breast cancer, with 45–50% of primary ductal carcinomas overexpressing this oncoprotein (Sutherland and Musgrove 2002). No reports have assessed the immnunoreactivity of cyclin D1 in cases of TVT. Therefore Rb protein and cyclin D1 appear to be novel markers of TVT in dogs.

c-Myc is an oncogene and plays an important role in proliferation and malignant transformation of human and animal cells (Amati et al. 1998; Bouchard et al. 1998; Dang, 1999). Most, if not all, types of human malignancy have been reported to display amplification and/or overexpression of this gene, although the frequency of these alterations varies greatly among different reports (Nesbit et al. 1999). Aberrant expression of c-myc also causes apoptosis (Evan et al. 1992; Shi et al. 1992). Studies have also shown that the *c-myc* gene regulates growth, both in the sense of cell size and in the context of tissue differentiation (Gandarillas and Watt 1997; Iritani and Eisenman 1999; Johnston et al. 1999; Schmidt 1999; Schuhmacher et al. 1999). In fact, it is now known that the c-myc gene participates in most aspects of cellular function, including replication, growth, metabolism, differentiation, and apoptosis (Packham and Cleveland 1992; Hoffman and Liebermann 1998; Dang 1999; Dang et al. 1999; Elend and Eilers 1999; Prendergast 1999; Liao and Dickson 2000). Immunoreactivity of c-myc was detected in the nucleus as well as cytoplasm in both the cases of TVT in the present study. Riuz et al (2005) have similarly reported strong nuclear immunoreactivity of c-myc in cases of TVT.

The diagnosis of mammary TVT in both the cases in the present study was supported by cytological and histopathological findings and was later confirmed on the basis of immunohistochemistry. Although TVT has been reported at aberrant sites such as nasal or oral cavities, skin, and rectum and more rarely on the lips, oral mucosa, and peritoneum, or in organs such as the tonsils, eye, liver, spleen, kidney, lung, and musculature (Park et al. 2006), yet there are no reports of TVT involving the mammary glands of dogs, and this seems to be the first report of primary mammary TVT in dogs.

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