Telomerase Reverse Transcriptase (TERT) Expression in Canine Mammary Tissues: A Specific Marker for Malignancy?

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Abstract. *Background: Telomerase reverse transcriptase (TERT), the catalytic subunit of the enzyme telomerase, is expressed in virtually all human tumors. Telomerase activity has also been reported in the majority of canine tumors and dogTERT also correlates with the enzyme activity. Materials and Methods: DogTERT expression in normal and malignant mammary tissues was investigated by immunohistochemistry and RT-PCR. Results: Using a highly specific TERT antibody in canins for the first time, immunoreactivity was identified in 46/50 malignant tumors, 26/50 adjacent to the tumor mammary tissues and 0/4 healthy mammary tissues. Two patterns of immunostaining were observed: cytoplasmic and concomitant nuclear and cytoplasmic. DogTERT mRNA was detected in 48/50 malignant tissues, 44/50 adjacent mammary tissues and in 2/4 healthy mammary tissues. Conclusion: The observation that normal canine mammary epithelium expresses TERT challenges the conventional view that this gene is repressed in somatic and activated in malignant cells and supports the notion that dogTERT may not be a useful marker for canine mammary cancer.*

Normal cells have a precise lifespan and they multiply until a critical point in cell division. After that, they are submitted to non-reversible cell arrest and undergo cellular senescence. On the other hand, cancer cells escape such restraint and multiply independently, a fact that can lead to increased cell division and uncontrolled proliferation. It is now well-established that the limited proliferation potential of normal cells is partially controlled by telomeric DNA.

Telomeres are specialized DNA-protein structures located at the ends of linear chromosomes (1). In mammalian cells, the telomere sequence consists of tandem repeats of the hexanucleotide motif TTAGGG (1). Telomeres shorten with each round of DNA replication, due to the inability of DNA polymerase to replicate the extreme ends of linear chromosomes. This leads to progressive telomeric attrition, which triggers cell growth arrest and cellular senescence (2, 3). Cancer cells have evolved mechanisms to circumvent telomere shortening, thereby bypassing senescence and allowing continuation of proliferation. The enzyme that plays a crucial role in this process by elongating telomeric sequences is a ribonucleoprotein complex, known as telomerase. The activity of telomerase is normally down-regulated in somatic cells, whereas it is up-regulated in actively dividing cells, such as malignant cells, a fact that suggests a key role of telomerase in tumorigenesis (4).

Telomerase consists of an RNA component (telomerase RNA, TR), a reverse transcriptase subunit (telomerase reverse transcriptase, TERT) and associated proteins (5, 6). TR anneals to the telomeric sequence TTAGGG and acts as a template for telomeric DNA synthesis, whereas TERT catalyzes the addition of telomeric repeats onto the chromosome ends. The TR subunit is essential, but not sufficient for telomerase activity, since it is expressed in all tissues regardless of telomerase activity (7). TERT, on the other hand, constitutes the catalytic subunit of the enzyme, since its expression is limited only to cells with telomerase activity (8). TERT expression is repressed in most normal somatic tissues, but it is elevated in the majority of human tumors (9). Thus, TERT has been proposed as a diagnostic and prognostic marker for a wide range of malignancies (10, 11).

During the last few years, there has been vast scientific interest in the use of telomerase as a target for cancer therapy. A great disadvantage, however, in telomerase-based studies remains the lack of a reliable animal model. The widely used transgenic mouse is not suitable in this case, since there are significant differences in both telomere maintenance and telomerase biology between mice and humans. Instead, accumulating evidence supports the dog as a more appropriate animal model in the case of telomerase (12, 13). The tissue distribution of telomerase activity in the dog is now known to

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be similar to that in humans, where it is largely confined to cancer cells or cells with high proliferative potential and absent from most normal somatic tissues (13). The dog has telomeres comparable in size to those in humans and dogTERT has higher homology to hTERT than any TERT protein from other species. Furthermore, the canine cancer subject represents a useful model for studying human carcinogenesis in general, since canine and human tumors share many similarities in terms of histopathology, biological behavior and response to therapy (13).

Telomerase activity has been reported in the majority (>90%) of canine tumors (12-17) and dogTERT expression also appears to correlate with the enzyme's activity (18, 19). The most commonly used method for the detection of telomerase activity in the dog has been the telomeric repeat amplification protocol (TRAP) (9). This assay, however, has many limitations, since it requires both enzymatically active specimens and good RNA integrity. Furthermore, TRAP does not allow identification of the cells expressing the enzyme. In a TRAP-positive sample, it is not known whether the telomerase activity originates from cancer cells, stem cells, highly proliferating cells, or lymphocytes. Recently, the cloning of canine TERT (18) has enabled the use of RT-PCR protocols for the detection of *TERT* mRNA expression. Furthermore, canine TERT has in many cases been detected immunohistochemically using commercially available anti-human TERT (hTERT) antibodies (19-24). Despite the fact that immunodetection of telomerase is now being recognized as a necessary approach to precisely elucidate its role in senescence and tumorigenesis (25), only a few antibodies directed against telomerase epitopes have been developed and their specificities are still under question. Recently, the mouse monoclonal antibody NCL-hTERT, widely used in both human and canine immunohistochemical studies, has been clearly demonstrated not to recognize hTERT protein, but either a protein known as nucleolin or a nucleolin-related protein (25). Nucleolin interacts with telomerase (26) and shows an almost identical pattern of expression with TERT (25). Among five commercial antibodies examined, the only one that specifically interacted with hTERT was the rabbit polyclonal antibody RCK-hTERT (25) which recognizes an epitope that resides between amino acids 1104-1123 of hTERT.

Based on the above observations, dogTERT expression in malignant mammary tumors and normal mammary tissues was investigated using immunohistochemistry and RT-PCR. The antibody RCK-hTERT was used for the first time to detect canine TERT protein and its subcellular localization.

Materials and Methods

Animals and tissue samples. Fifty bitches (5-15 years of age) with malignant tumors of the mammary gland and four clinically healthy bitches with no history of cancer or infectious diseases were used in this study. For each cancer subject, tissue specimens were taken during surgery from both the tumor and a site 2-5 cm from the visible tumor margin (adjacent to the tumor clinically normal mammary tissues). For each client-owned healthy dog, tissue samples were taken, with the owner's permission, from the mammary gland during routine ovariohysterectomy. All the tissue specimens were either fixed in 10% neutral-buffered formalin for histopathology and immunohistochemistry, or immersed in an RNAlater solution (Ambion, Austin, TX, USA) and kept at –80˚C for RNA extraction and RT-PCR. Normal canine testis and brain tissues were used as positive and negative controls, respectively.

Histopathology. Formalin-fixed tissues were embedded in paraffin wax, sectioned at 4-6 μm, and stained with hematoxylin and eosin (HE). According to the World Health Organization (WHO) criteria, the tumor tissues were classified as 16 complex carcinomas, 24 simple carcinomas, 3 special type carcinomas, 6 sarcomas and 1 carcinosarcoma. The histopathological lesions were scored with the following criteria according to the grading system of canine mammary neoplasias (27): grade I, score 3-5; grade II, score 6-7 and grade III, score 8-9. Accordingly, the tumor tissues were classified as: 15 grade I, 26 grade II and 9 grade III. No histological changes were detected in any of the adjacent normal mammary tissues or in the normal tissues obtained from the healthy dogs.

Immunohistochemistry. Immunohistochemical detection of dogTERT was performed using two different antibodies, the mouse monoclonal antibody (clone 44F12) NCL-hTERT (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) and the rabbit polyclonal antibody RCK-hTERT (Rockland Immunochemicals Inc., Gilbertsville, PA, USA). Standard avidin-biotin complex procedures were applied (ABC kit, peroxidase standard Vectastain; Vector Laboratories, Burlingame, CA, USA). Formalin-fixed tissues on positively charged slides were deparaffinized and rehydrated. For the NCL-hTERT antibody, antigen retrieval was performed with microwave heat in a high pH solution (Dako, Glostrup, Denmark) for 10 min at 600 W. The sections were incubated with the primary antibody NCL-hTERT (diluted 1:25) overnight at 4˚C, followed by a secondary horse anti-mouse biotinylated antibody (Vector) (diluted 1:125) for 30 min at room temperature. For the RCK-hTERT antibody, the antigens were retrieved with microwave heat in a pH 6.0 citrate buffer solution (Merck, Darmstadt, Germany) for 10 min at 700 W. The sections were incubated with the primary antibody RCK-hTERT (diluted 1:500) overnight at 4˚C, followed by a secondary goat anti-rabbit biotinylated antibody (Vector) (diluted 1:250) for 30 min at room temperature. In both procedures, the signal was detected with 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA) and the tissues were counterstained with Mayer's hematoxylin. To evaluate hTERT antibody specificity in control tissues, the primary antibody was omitted from the above described protocols.

Semi-quantitative assessment of the immunoreactive cells was performed by two independent observers. At least five high power fields (×400) were selected throughout each section, from both the periphery and the center of the tissue, and a minimum of 1,000 cells per case were counted. The number of positive cells was expressed as a percentage of the counted nuclei. The following scoring system was used: score 0: no staining (0% positive cells); score 1: mild staining (1-10% positive cells); score 2: moderate staining (11-30% positive cells) and score 3: strong staining (>30% positive cells).

Table I. *Immunohistochemistry patterns observed using the two anti-hTERT antibodies.*

	Tumor tissue				Adjacent normal tissue				Normal tissue			
	N	C	N/C	P	N	C	N/C	P	N	C	N/C	P
NCL-hTERT Ab	22	10	15	47	9	9	12	30	-	-	1	1
RCK-hTERT Ab	-	29	17	46	-	13	13	26	-	-	-	0

N: Nuclear staining; C: cytoplasmic staining; N/C: nuclear and cytoplasmic staining; P: total number of positive samples.

RNA extraction and cDNA synthesis. The total RNA was extracted from the tissue samples using the NucleoSpin Total RNA Isolation kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The RNA concentration and quality were determined spectrophotometrically, and the samples were stored at –80˚C until use. Reverse transcription was carried out using the SuperScript RT-PCR system (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. One μg of total RNA was used as the starting material for cDNA synthesis.

PCR. Primers 2084F and 2586R, which anneal at positions 2084-2100 and 2567-2586 of the dog*TERT* gene (GenBank accession number AF380351) respectively, were used to amplify a 503 bp DNA fragment according to the method of Nasir *et al*. (18). The PCR products were subsequently subjected to a nested PCR amplification, using the following internal primers designed in this study: 2101-F, 5'-TACGCATACGGGCC CAGAAT-3' and 2569-R, 5'-TCCATGTCCCCGTAGCA C-3'. The primers anneal at positions 2101-2120 and 2551-2569 of dog*TERT*, respectively and amplify a DNA sequence of 469 bp. The PCR conditions were optimized using normal testis as a positive control and normal brain as a negative control. The first PCR amplification was performed in 25 μl reaction mixtures containing 1.5 μl cDNA, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 0.4 μM of each of the two primers and 1 unit Taq DNA polymerase (Invitrogen). The temperature cycling protocol on a Techgene Thermal Cycler (Techne Limited, Cambridge, UK) consisted of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s and extension at 72˚C for 40 s. The cycling was repeated 35 times. Each PCR reaction was initiated with a 5 min denaturation at 94˚C and terminated with a 5 min extension at 72˚C. The same conditions were used for the nested PCR with the following differences: 1.5 μl of the first PCR product was used as the starting material and primer annealing took place at 58˚C. PCR amplicons were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and visualized on a UV transluminator.

In order to verify good RNA integrity, all the cDNA samples were also subjected to PCR amplification of the canine *β-actin* housekeeping gene, using previously published primers (28). The PCR conditions were the same as those in the nested PCR.

Results

Immunohistochemical detection of dogTERT protein. Using the NCL-hTERT antibody, immunoreactivity was detected in 47/50 tumor tissues (94%), 30/50 adjacent to the tumor normal tissues (60%) and 1/4 normal tissues (25%) (Table I). Three patterns of staining were observed: nuclear, cytoplasmic, and both nuclear and cytoplasmic. Signal intensity in all cases was mild to strong, not uniformly distributed and varied from case to case.

Using the RCK-hTERT antibody, dogTERT protein was identified in 46/50 tumors (92%; 12/15 grade I, 25/26 grade II and 9/9 grade III), 26/50 adjacent to the tumor normal tissues (52%) and 0/4 normal tissues (Table I). This antibody revealed only two patterns of staining, either cytoplasmic, or both nuclear and cytoplasmic. No strictly nuclear localization was detected in any of the cases. Out of the 46 dogTERT-positive tumor tissues, 17 had both nuclear and cytoplasmic expression (Figure 1 A, B), whereas 29 exhibited only cytoplasmic staining (Figure 1C). Out of the 26 positive adjacent normal tissue samples, 13 showed cytoplasmic (Figure 1 D) and 13 nuclear and cytoplasmic staining. In all cases, the RCK-hTERT antibody gave a strong and uniformly distributed signal. Overall, 25 cases were positive in both malignant and adjacent normal tissues and 3 were negative in both. In 21 cases, dogTERT was detected only in the tumor, and in one case the protein was identified only in the adjacent normal tissue. Adjacent normal tissues, when positive, exhibited much stronger nuclear and cytoplasmic staining than did the neoplasias. In all the sections, dogTERT was detected only in the epithelial cells, no immunoreactivity was identified in any of the neighboring cells (macrophages, fibroblasts, endothelial cells). The mammary tissues from healthy bitches exhibited no immunoreactivity (Figure 1 E). Normal testicular tissues, used as positive controls, had both nuclear and cytoplasmic immunostaining (Figure 1 F), whereas no signal was seen in any of the normal brain tissues used as negative controls.

Detection of dogTERT mRNA. In order to achieve increased sensitivity and specificity in the detection of dog*TERT* mRNA, a nested RT-PCR method was developed. Dog*TERT* mRNA was identified in 48/50 malignant tissues (96%), 44/50 adjacent normal tissues (88%) and in 2/4 normal healthy tissues (Figure 2 A). All the samples were also analyzed for the canine *β-actin* gene in order to verify RNA quality (Figure 2 B). Out of the 50 canine subjects, 42 were

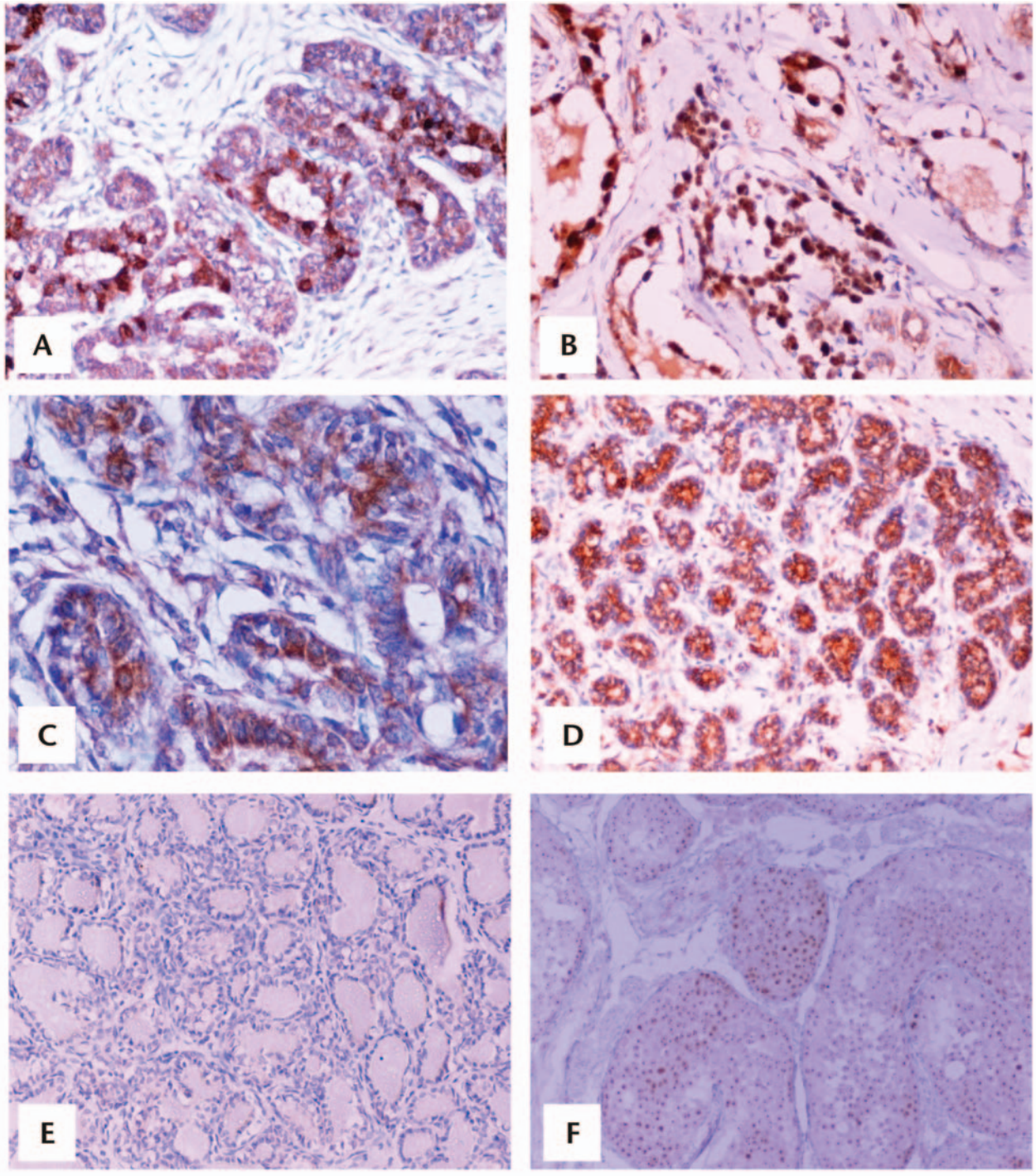

Figure 1. *Localization pattern of immunohistochemical staining using RCK-hTERT antibody. A, Nuclear and cytoplasmic staining in a complex carcinoma (×20). B, Nuclear and cytoplasmic staining in a tubulopapillary carcinoma (×20). C, Cytoplasmic staining in a simple tubulopapillary carcinoma (×40). D, Cytoplasmic staining in adjacent to tumor normal tissue (×20). E, No immunostaining in normal mammary tissue from a healthy dog (×20). F, Nuclear and cytoplasmic staining in normal canine testis (×20).*

positive in both tumor and adjacent normal tissue, 6 were positive only in the tumor, and 2 were positive only in the adjacent normal tissue.

Out of the 50 tumor tissues, 45 were positive by both RT-PCR and immunohistochemistry (using the RCK-hTERT antibody) and 1 was negative by both methods. Three

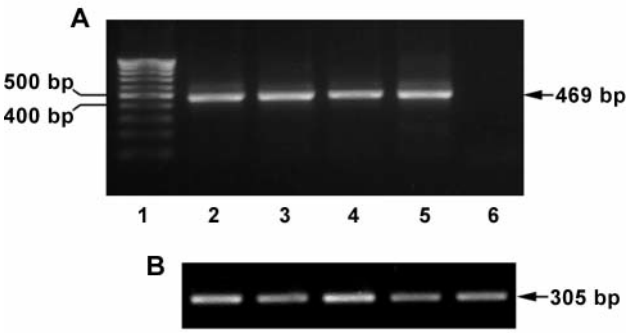

Figure 2. *A, Detection of dogTERT mRNA with nested RT-PCR. Lane 1: 100 bp DNA molecular weight markers; lanes 2, 3: a tumor and its corresponding adjacent normal tissue, respectively; lane 4: a positive normal tissue from a clinically healthy dog; lanes 5, 6: a tumor and its corresponding adjacent normal tissue, positive and negative for dogTERT mRNA, respectively. B, Detection of dog β-actin mRNA with RT-PCR. Lanes 2-6, as in (A).*

samples were positive only by RT-PCR and 1 was positive only by immunohistochemistry. However, more discrepancies between the two methods were observed in the adjacent normal tissues. Out of the 50 cases, 22 were positive and 2 were negative by both methods, 22 were positive only in RT-PCR and 4 were positive only in immunohistochemistry.

To further confirm that the amplified products were indeed dog*TERT* DNA sequences, three PCR amplicons (one from a tumor sample, one from an adjacent normal tissue and one from a normal healthy tissue) were subjected to DNA sequencing. The sequences obtained exhibited 100% homology between them and 99.4% homology with dog*TERT* gene (GenBank accession number AF380351; data not shown).

Discussion

Immunodetection with RCK-hTERT was observed in 92% of the tumor tissues, 52% of the histologically normal tissues adjacent to the tumor and in none of the normal mammary tissues. Similar immunoreactivity was also identified with the NCL-hTERT antibody. This was not surprising since nucleolin and telomerase are known to interact and share a similar pattern of expression (26). Additionally, the ability of nucleolin to bind to the human telomeric DNA sequence TTAGGG has also been reported (29, 30).

Three intracellular patterns of TERT protein expression, nuclear, cytoplasmic, and both nuclear and cytoplasmic, have been reported. In the present canine tissues, no strictly nuclear immunoreactivity was observed with the specific anti-hTERT antibody. This finding was in agreement with data presented by Colitz *et al*. (22) who identified no nuclear localization of dogTERT in canine lens epithelial cells, using a different specific anti-TERT antibody. After transcription, *TERT* mRNA shuttles from the nucleus to the cytoplasm, where it is translated, and the protein produced is transferred back into the nucleus. Nuclear detection of TERT is a strong indication of active telomerase enzyme and ongoing cell proliferation. The roles of TERT in the cytoplasm have not as yet been clarified. Some reports, however, implicate cytoplasmic TERT in the inhibition of apoptosis (31). Many studies have shown that intracellular translocation of hTERT from the cytoplasm to the nucleus constitutes one of the mechanisms involved in telomerase activation (32-34) and that nuclear localization is associated with phosphorylated hTERT protein (35). Jagadeesh *et al*. (34) demonstrated that inhibition of hTERT phosphorylation abolished nuclear staining with a parallel increase of cytoplasmic staining. It was thus suggested that reduced phosphorylation either renders hTERT unable to bind to its nuclear translocators or forces the protein to move from the nucleus to the cytoplasm.

In the case of malignant mammary tissues, the results of the RT-PCR for the detection of canine *TERT* mRNA expression were in good agreement with those obtained by immunohistochemistry using the RCK-hTERT antibody (positive samples 96% vs. 92%, respectively). However, a great discrepancy between the two methods was observed in the histologically normal tissues adjacent to the tumor. While only half of the cases were positive in immunohistochemistry, 88% of these samples exhibited dog*TERT* mRNA expression by RT-PCR. Furthermore, two out of the four normal mammary tissues obtained from clinically healthy dogs were positive in RT-PCR, whereas no dogTERT protein was detected by immunohistochemistry in the corresponding paraffin sections. This might be attributed to the higher sensitivity of the nested PCR technique, which can detect very low amounts of mRNA transcripts. The possibility that dog*TERT* mRNA detected in these cases may in fact not be expressed by tissue epithelial cells but by activated infiltrating lymphocytes present in these tissues could also not be excluded.

Funakoshi *et al*. (15) reported no telomerase activity in either canine normal mammary tissues or canine mammary gland hyperplasias. Two other studies, however, have demonstrated telomerase activity in normal mammary gland and fibrocystic mastopathies, but the number of samples analyzed was small (16, 21) and the NCL-hTERT antibody was used in the latter report. The present finding that canine normal mammary epithelium expressed TERT contradicts the conventional view that TERT expression is repressed in somatic cells and activated in neoplastic cells, but it was in absolute agreement with findings from human mammary tissues. Using *in situ* hybridization in a large series of human tissue samples, Liu *et al*. (36) demonstrated that both normal breast ductal-lobular units and adjacent ductal carcinoma *in situ* (DCIS) expressed high levels of *hTERT* mRNA. In fact, *hTERT* mRNA levels were significantly higher in normal cells compared to DCIS. In agreement with these observations, using real-time RT-PCR, Hines *et al*. (37)

showed that hTERT is expressed in both normal and malignant breast tissues and that approximately 75% of breast tumors express *hTERT* mRNA levels equal to or less than those within normal breast tissues. This is consistent with the present observation that the normal tissues adjacent to the tumor, when positive, exhibited much stronger immunostaining than did the neoplasias. However, it has to be determined whether such positivity also reflects increased telomerase activity. It is possible that TERT may be regulated at a post-transcriptional or even a post-translational level and give rise to a non-active telomerase.

To our knowledge, this is the first time that the specific anti-hTERT antibody RCK-hTERT has been evaluated in canine tissues, that a nested RT-PCR method has been applied for the detection of dog*TERT* mRNA in canine mammary tissues and compared to immunohistochemistry, and that matched tumor and adjacent normal tissue specimens have been analyzed for canine TERT expression. Overall, the findings support the notion that TERT may not be a useful marker for canine mammary cancer.

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