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MITOSIS AND APOPTOSIS IN CANINE CUTANEOUS HISTIOCYTOMA AND TRANSMISSIBLE VENEREAL TUMOUR

T. GUVENC[1*], M. HALIGUR[1], M. N. ORMAN[2] and R. HAZIROGLU[1]

[1]Pathology Department, Veterinary Medicine Faculty, Ankara University, 06110 Diskapi, Ankara, Turkey; [2]Biostatistics Department, Veterinary Medicine Faculty, Ankara University, Ankara, Turkey

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Cell proliferation and apoptosis in canine cutaneous histiocytomas and transmissible venereal tumours were examined in twenty cases. The Ki-67 immunohistochemistry and Tunel methods were used to detect mitotic activity and apoptosis, respectively. The number of Ki-67 immunoreactive cells was 11.65 (± 1.1706) in canine cutaneous histiocytomas and 17 (± 2.1751) in transmissible venereal tumours. The mean values of apoptotic cells for canine cutaneous histiocytomas and transmissible venereal tumours were 13.25 (± 1.8758) and 8.52 (± 1.1007), respectively. It was considered that mitotic activity and apoptotic indices were useful in differentiation of canine cutaneous histiocytomas and transmissible venereal tumours. The correlation values for canine cutaneous histiocytomas and transmissible venereal tumours were 0.359 (± 0.330) and –0.232 (± 0.344), respectively. No significant ($P > 0.05$) correlation was found between mitosis and apoptosis in these two tumour types.

Key words: Dog, differential diagnosis, canine cutaneous histiocytoma, canine transmissible venereal tumour, Ki-67, Tunel

Canine transmissible venereal tumour (CTVT) is widely recognised as a naturally occurring neoplastic disease. It is transmitted by coitus. CTVT forms a round cell neoplasm of the mucous membranes of the external genitalia of both sexes within the Canidae family. CTVT also occurs in the oral, nasal, and conjunctival mucosa and in the skin (Nielsen and Kennedy, 1990; Karademir et al., 1998; Gonzales et al., 2000). Canine cutaneous histiocytoma (CCH) is characterised by the benign skin growth found only in dogs. CCH consists of uniform sheets of cells, infiltrating the dermis and subcutis. Although rapid growth and high mitotic index suggest malignancy, CCH is a benign tumour in all aspects. CCH does not metastasise, and the great majority of tumours spontaneously regresses (Pulley and Stannard, 1990; Karademir et al., 1998). CCH and CTVT also exhibit different biological and epidemiological characteristics (Nielsen and Kennedy, 1990; Pulley and Stannard, 1990). However, the differential diagnosis

[*]Corresponding author; E-mail: guvenc@veterinary.ankara.edu.tr; Fax: 0090 312 316 44 72

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of CCH and CTVT, with conventionally stained tissue sections, may be difficult because of morphological similarities between these two tumour types (Nielsen and Kennedy, 1990; Pulley and Stannard, 1990; Karademir et al., 1998).

In some tumours, a direct relationship between slower growth rates and higher apoptotic indexes has been observed (Kasagi et al., 1994; Shigihara and Lloyd, 1997). Additionally, differences in the apoptotic and mitotic ratios were reflected in the clinical behaviour of certain tumour types, such as basal cell carcinoma and melanoma (Mooney et al., 1995).

The assessment of cell proliferation in a neoplasm may provide useful diagnostic and prognostic information (Sarli et al., 1994). The proliferation-associated nuclear antigen, Ki-67, is one of the most widely used markers for determining cell proliferation. Ki-67 indices could be exploited to assess the proliferative activity of CTVT and CCH (Griffey et al., 1999; Roels et al., 1999).

In the literature, CCH and CTVT cases have not been compared using the parameters of Ki-67 immunoreactivity and apoptosis. Therefore, it is essential to investigate whether or not cellular proliferation and apoptosis can be used as diagnostic tools and if there is a correlation between these two criteria.

Materials and methods

Ten cases of each CTVT and CCH were chosen from the archives of the Pathology Department, Veterinary Medicine Faculty, Ankara University. Following routine staining with haematoxylin-eosin, specimens were diagnosed as being either CCH or CTVT.

Ki-67 immunohistochemistry

The sections were dewaxed in xylol and rehydrated in decreasing alcohol series. Slides were incubated in 3% hydrogen peroxide for 30 min to block endogenous peroxidase. Sections were then rinsed in PBS, and immersed in antigen retrieval solution (DAKO A/S), pH 6.0, for 20 min at 90 °C. Slides were then allowed to cool down at room temperature. Ki-67 immunohistochemistry was performed with the clone MIB1 (DAKO A/S) using streptavidin-biotin peroxidase technique with a commercial kit, according to the manufacturer's instructions (LSAB 2, DAKO A/S).

TUNEL method

Sections were stained with an *in situ* apoptosis detection kit (Apoptacs, R&D systems). Dewaxed and rehydrated tissue sections were treated with cytonin (R&D systems) for 20 min and rinsed twice in deionised water for 2 min each. Sections were incubated in 3% hydrogen peroxide for 5 min to block en-

dogenous peroxidase activity. They were then washed in PBS for 1 min and immersed in TdT labelling buffer for 5 min. Samples were incubated in the labelling reaction mixture for 1 h at 37 °C in a humidity chamber. Slides were then immersed in the TdT stop buffer for 5 min to stop the labelling reaction. After 2 rinses in PBS, sections were incubated with anti-BrdU antibody for 1 h at 37 °C. After slides were washed 3 times with PBS, they were incubated with streptavidin-HRP solution for 10 min. Then samples were washed 3 times in PBS. DAB was used as a chromogen and slides were counterstained with Mayer's haematoxylin.

Scoring and statistical analysis

All immunostained sections were examined at a magnification of ×400. The Tunel signals and MIB-1 immunostained nuclei were scored by counting 1000 cells in randomly selected fields per histological section. Every stained nucleus was considered positive regardless of intensity of staining. The Student's *t*-test was employed to determine statistical differences between CTVT and CCH. Pearson's correlation coefficient was calculated between Ki-67 and apoptosis for both tumour types. A P value of < 0.05 was considered statistically significant.

Result

Ki-67 immunohistochemistry

The nucleus of tumour cells positive for Ki-67 was light to dark brown in colour (Figs 1–2). The Ki-67 negative nuclei were light blue. The values for Ki-67 activity ranged from 9 to 17.2% in CCH and from 5 to 26.7% in CTVT (Fig. 3). The number of Ki-67 immunoreactive cells was 11.65 (± 1.1706) in CCH and 17 (± 2.1751) in CTVT.

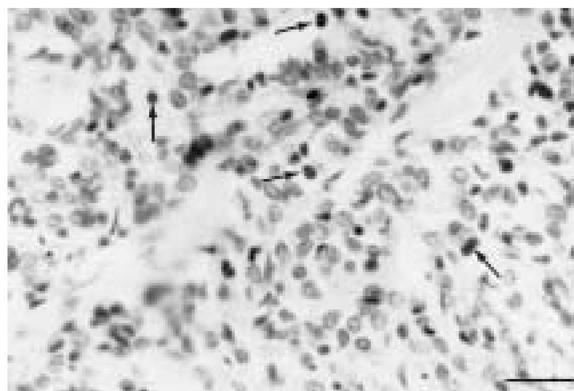

Fig. 1. CCH stained for proliferating cells with MIB-1 antibody (arrows). Biotin-streptavidin/DAB immunohistochemistry, counterstain haematoxylin. Bar: 40 μm

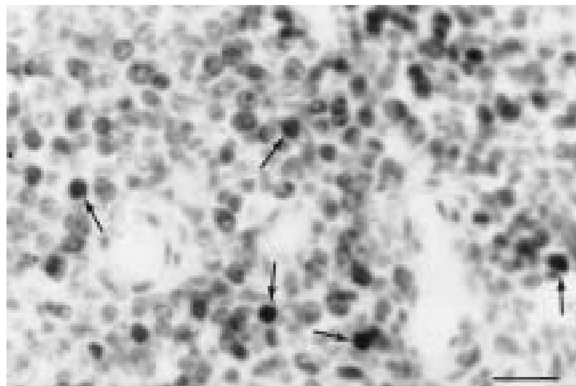

Fig. 2. CTVT stained for proliferating cells with MIB-1 antibody (arrows). Biotin-streptavidin/DAB immunohistochemistry, counterstain haematoxylin. Bar: 40 µm

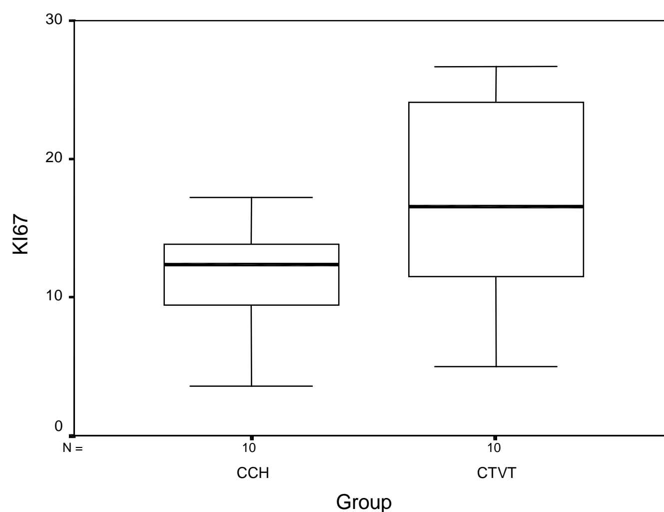

Fig. 3. Distribution of Ki-67 immunoreactive cells of the tumours in box-plots

Tunel reaction

Tunel signals were observed in both CCH and CTVT. By the Tunel method, nuclei in apoptotic cells appeared brown in colour (Figs 4–5). Two different Tunel positive staining reactions were seen in the tumour cells; diffuse nuclear staining (DNS) and nuclear and cytoplasmic staining (NCS). In the cases of CCH, both DNS and NCS were observed. In CTVT cases, only DNS was seen. The number of apoptotic nuclei was 3.3 to 23.2% in CCH and 4 to 14.8% in CTVT (Fig. 6). The mean values for CCH and CTVT were 13.25 (± 1.8758) and 8.52 (± 1.1007), respectively.

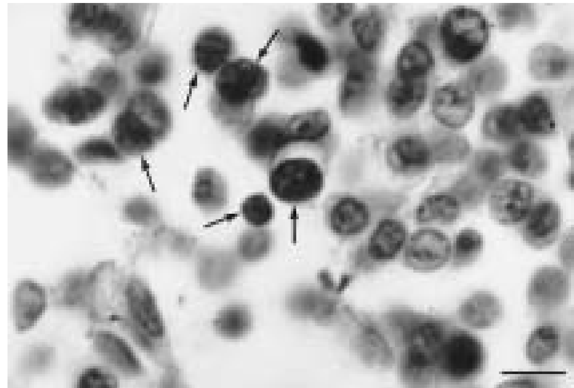

Fig. 4. CCH stained for apoptotic cells (arrows). Tunel method, counterstain haematoxylin. Bar: 15 µm

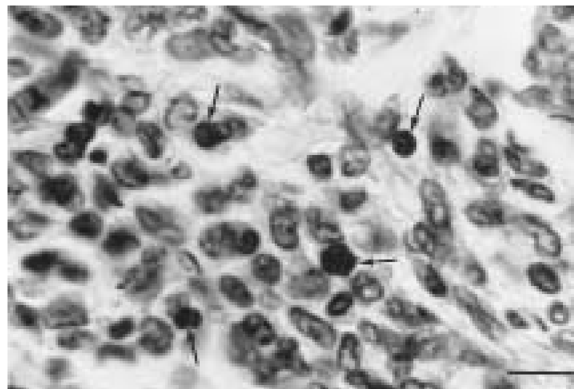

Fig. 5. CTVT stained for apoptotic cells (arrows). Tunel method, counterstain haematoxylin. Bar: 15 µm

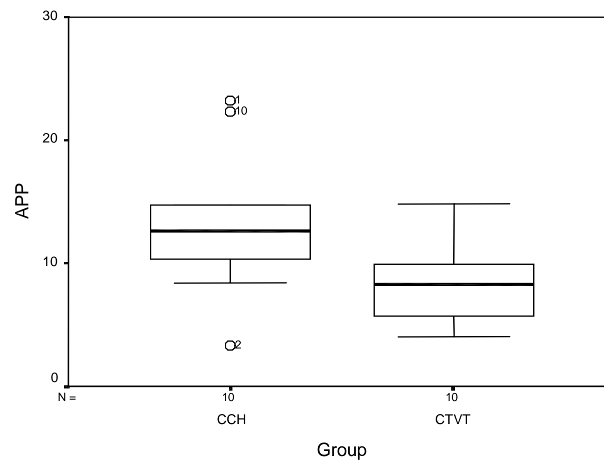

Fig. 6. Distribution of apoptotic nuclei of the tumours in box-plots

Statistical analysis

Statistical analysis revealed that Ki-67 indices and apoptotic indices were significant to differentiate between CCH and CTVT ($P < 0.05$ for Ki-67 and apoptosis). The Ki-67/apoptotic index ratios for CCH and CTVT were 0.87 and 1.99, respectively. On the other hand, nonsignificant correlation values were found between Ki-67 and apoptosis ($P > 0.05$). The correlation values for CCH and CTVT were 0.359 (± 0.330) and –0.232 (± 0.344), respectively.

Discussion

Our results indicate that Ki-67 immunoreactivity and apoptotic cell death can be used in the differential diagnosis of CCH and CTVT. Additionally, Ki-67 immunoreactivity is not correlated with apoptotic cell death in these tumour types.

Several methods are used routinely for evaluating cellular proliferation markers in tumours (Sarli et al., 1994; Löhr et al., 1997; Roels et al., 1999). Mitotic indices and immunohistochemical labelling for cell proliferation associated proteins are the most commonly used methods (Löhr et al., 1997; Griffey et al., 1999). We opted for the immunohistochemical method for assessment of a proliferation associated antigen, called Ki-67. In this study, the mitotic figures were easily recognised at low magnification in the clone MIB-1 labelled sections. This property makes MIB-1 reactivity a useful marker for rapid determination of mitotic counts and distributions in given tumours. Our study confirmed that Ki-67 immunoreactivity, with clone MIB-1, can be easily standardised (Löhr et al., 1997).

In our study, the number of Ki-67 immunoreactive cells were 11.65 (± 1.1706) in CCH and 17 (± 2.1751) in CTVT. Martin De Las Mulas et al. (1999) reported that in CCH cases the average mitotic count was 10.6 (± 2.5), but in the present study the mean mitotic count was higher. The mean values of apoptotic nuclei were 13.25 (± 1.8758) in CCH and 8.52 (± 1.1007) in CTVT. It was reported that mean apoptotic cell counts were 3.7 and 8.73 in CCH and CTVT cases, respectively (Martin De Las Mulas et al., 1999; Gonzales et al., 2000). Our finding on CTVT is in agreement with a recent study (8.73 versus 8.52; Gonzales et al., 2000). However, in our study, the average number of apoptotic cells in CCH was 3-fold higher than those reported by Martin De Las Mulas (1999). Our findings indicate that apoptotic cell death can easily be used as a diagnostic tool for identification of these tumours.

It has been reported that in the majority of benign tumours, there is a negative relationship between slower growth rates and higher apoptotic indexes (Kasagi et al., 1994; Shigihara and Lloyd, 1997). In contrast, Martin De Las Mulas (1999) recorded low apoptosis and high mitotic counts, and a positive correlation between the two parameters in CCH. In the present report, however, neither CCH nor CTVT cases have significant correlation values between Ki-67

and apoptosis. The differences in the results of these studies might have been due to different staining techniques used, and the variability of case numbers, counting techniques or the tumours being at the stage of regression or proliferation.

In summary, Ki-67 indices and apoptosis can be used as a diagnostic tool in the differential diagnosis of CTVT and CCH, and there is no correlation between apoptosis and Ki-67 indices in these tumours.

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