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Research Article

PATHOMORPHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES ON TRANSMISSIBLE VENEREAL TUMOURS IN DOGS

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ABSTRACT

The present study reports pathomorphological changes and immunohistochemical expression of pancytokeratin (PCK), vimentin and proliferating cell nuclear antigen (PCNA) in transmissible venereal tumours (TVT) in dogs. Out of 32 cases studied, 3 (9.37%) cases were diagnosed as TVT. These were noticed in a 4, 6 and 8 years old male Bully, non-descript female and male German shepherd dogs with presence of tumourous growths (irregular, multi-nodular to cauliflower-like in shape) in prepuce, vagina and penis, respectively. Cytological examination of Giemsa stained impression smears revealed round cells with faint basophilic cytoplasm and characteristic punctate cytoplasmic vacuoles. Histopathological examination showed round to oval neoplastic cells arranged in cord-like fashion and separated by thin fibrous connective tissue strands. Neoplastic cells revealed large round to oval vesicular nuclei, foamy cytoplasm and numerous mitotic figures. Mean values of mitotic index and AgNOR count in all TVT cases were 3.06 and 2.42, respectively. Higher mitotic index and AgNOR count indicated high proliferation rate and aggressiveness of tumour. No immunoreactivity for PCK noticed in all TVT cases suggested the non-epithelial origin of neoplastic cells. Mild to moderate intracytoplasmic immunostaining for vimentin in about 90-95% neoplastic cells was observed which indicated the mesenchymal origin of neoplastic cells. Proliferating neoplastic cells revealed moderate to strong nuclear PCNA immunoreactivity and PCNA index was 20.9%, 19.2% and 40.0%. It may be concluded that estimation of mitotic index, AgNOR count and immunohistochemical expression of tumour markers proves important adjunct tools for diagnosis, determination of the biological behaviour and origin of tumour.

Key words: Proliferating cell nuclear antigen, Transmissible venereal tumour, Vimentin

In dogs, transmissible venereal tumour (TVT) is most common genital tumour. It mainly affects the external genitalia and occasionally, the internal genitalia (Martins *et al.*, 2005).

Cytological examination may be used for diagnosis of various types of tumours, but histological examination remains the gold standard for differentiation between benign and malignant tumours. Tumour markers expression profile play an important role in differentiating the histologically similar tumours particularly undifferentiated type, origin of neoplastic cells and predict therapeutic response of neoplastic diseases (Mukaratirwa, 2005). Cytokeratins are present within the cytoplasm of all epithelial cells and their tumours (Babu *et al.*, 2012). Vimentin is an intermediate filament, which is most often used as a marker for mesenchymal derived cells or cells which are undergoing an epithelial to mesenchymal transition during both normal development and metastatic progression (Kim *et al.*, 2014).

Among several proliferative tumour markers used to estimate the proliferation rate of neoplastic cells, most commonly used histochemical proliferation marker is argyrophilic nucleolar organizer region (AgNOR) (Lohr *et al.*, 1997). Proliferating cell nuclear antigen (PCNA) and Ki67 are important prognostic factors and determinants of aggressiveness of a tumours (Morris and Mathews, 1989; Mukaratirwa, 2005). Keeping above facts in view, the present study was conducted with an objective to study the pathomorphological changes along with immunohistochemical expression of pancytokeratin, vimentin and PCNA in TVT.

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MATERIALS AND METHODS

The present study was conducted on three cases of TVT out of thirty two cases, which were presented to Department of Veterinary Clinical Complex of Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar from July, 2017 to February, 2018.

Cytology: The impression smears were prepared from tumour samples and fixed with absolute methanol. The dried smears were stained with Giemsa and Toluidine blue stains (Luna, 1968). Then, these were examined under microscope for cytopathological evaluation.

Histopathology, mitotic index and AgNOR count

Representative tissue samples were fixed in 10% buffered formalin. The sections of 4-5 micron thickness were cut and stained with haematoxylin and eosin (H&E) (Luna, 1968). For evaluation of mitotic index, region of tumour section in H&E stained slides with highest overall mitotic activity was chosen. It was expressed as number of mitotic figures/10 high power fields (hpf) (Romansik *et al.*, 2007). AgNOR count was done as per method described by Crocker *et al.* (1989) with slight modifications. All AgNOR dots scattered in nucleus were counted without trying to resolve the intranucleolar dots. Counting of AgNOR dots was done in 100 consecutive nuclei and mean number of AgNOR dots per nucleus was calculated for each specimen.

Immunohistochemistry: Duplicate sections of 3-4 micron thickness of formalin fixed tissues were cut and taken on 3-aminopropyl-triethoxysilane coated slides. Sections were deparaffinized in xylene, then rehydrated through descending grades of ethanol (100, 90, 80, 70 and 50%) and distilled water. Antigen retrieval was performed by subjecting the tissue sections to microwave irradiation in a coplin jar containing 0.01M citrate buffer (pH 6.0) for 30 minutes (min.) Quenching of endogenous peroxidase activity was carried out by treating sections with 3% hydrogen peroxide (prepared in absolute methanol) for 15 min. The non-specific sites were blocked by 5% normal goat serum (Sigma Chemicals) prepared in phosphate buffered saline (PBS, pH 7.4) for 30 min. Immunostaining was carried out as per manufacturer's instructions with slight modifications. Primary mouse monoclonal antibodies; anti-pancytokeratin (clone PCK-26, Sigma), anti-vimentin (clone V9, Sigma) and anti-PCNA (clone PC10, Sigma) were used at 1:100, 1:400 and 1:400 dilutions, respectively. Dilutions were made in 1% bovine serum albumin (BSA, Sigma Chemicals, USA) prepared in PBS (pH 7.4). Secondary antibody and Extravidin peroxidase were used at 1:20 dilution in 1% BSA prepared in PBS. Coloured reactions were developed with 3-Amino-9-ethylcarbazole (Sigma Chemicals) as staining substrate and counter stained with Gill's haematoxylin (Sigma Chemicals). Sections were mounted in aqueous mounting medium. Negative and positive controls were used. Negative controls were covered with diluent only. Reddish brown to yellowish brown staining in the cytoplasm of neoplastic cells was considered positive immunostaining for PCK and vimentin, while brick red or reddish brown staining in nuclei of neoplastic cells was considered as positive for PCNA.

Scoring of PCK immunostaining was done according to Baghla *et al.* (2012) based on percentage of PCK positive cells i.e. 0 for no immunostaining, 1 for 1-25%, 2 for 26-50%, 3 for 51-75% and 4 for 76-100% positive stained cells. Scoring of immunostaining for vimentin was done according to Heller *et al.* (2005). The staining distribution was given scores 0 for no immunostaining; 1 for 1-25%, 2 for 26-50%, 3 for 51-75% and 4 for 76-100% positive stained cells. Scoring of PCNA immunostaining was done as per method described by Kumaraguruparan *et al.* (2006) based upon percentage of PCNA positive cells i.e. 0 for <5%, 1 for 5-25%, 2 for 26-50%, 3 for 51-75% and 4 for >75% positive cells. PCNA index was calculated as per the method described by Lokesh *et al.* (2014).

RESULTS AND DISCCUSSION

Out of 32 cases studied, 3 (9.37%) cases were diagnosed as TVT. The first case was observed in a 4 years old, male Bully dog. The affected dog showed multi-nodular tumourous growths in prepucial region (Fig. 1) and exhibited the clinical signs such as difficulty in protrusion of penis, blood in urine and spotting of blood. In second case, the growth was observed in 6 years old, non-descript female dog in vaginal mucosa and it appeared irregularly round. Third case was noticed in 8 years old, male German shepherd dog and it showed cauliflower-like growths at the base of penis (Fig. 2) and exhibited clinical signs such as blood in urine and spotting of blood. The occurrences of TVT in external genitalia in present study were similar to earlier workers (Birhan and Chanie, 2015). The cut surface appeared light pink to red in colour in all cases and consistency varied from soft to hard.

Cytological examination of Giemsa stained smears revealed round cells with faint basophilic cytoplasm, characteristic punctate cytoplasmic vacuoles and few neutrophils (Fig. 3). Smears did not reveal metachromatic granules with Giemsa and Toluidine blue stains, thereby differentiating it from mast cell tumour. Histologically, these were characterized by round to oval neoplastic cells arranged in cord-like fashion and separated by thin fibrous connective tissue strands (Fig. 4). Neoplastic cells revealed large round to oval vesicular nuclei, foamy cytoplasm and numerous mitotic figures (Fig. 5). Mitotic index/Mean AgNOR count in male Bully, non-descript female and male German shepherd dogs were 3.0/2.18, 2.7/2.01 and 3.5/3.08, respectively. AgNOR dots were large, round and appeared black in colour (Fig. 6). Mean values of mitotic index and AgNOR count in all TVT were 3.06 and 2.42, respectively.All cases revealed no immunoreactivity for PCK and assigned 0 score indicating non-epithelial origin of TVT. All cases revealed mild to moderate intracytoplasmic vimentin immunostaining in 90-95% neoplastic cells (Fig. 7) and given 4 score for vimentin indicating mesenchymal origin of TVT. PCK and vimentin are the diagnostic markers which help in confirmation and differentiation of tumours. Proliferating neoplastic cells revealed moderate to strong nuclear immunoreactivity for PCNA (Fig. 8). PCNA immunoreactivity score in two cases was 1, while in third case it was 2. PCNA index was 20.9%, 19.2% and 40.0%. PCNA is a proliferating marker which helps in assessing the aggressiveness of a tumour and plays an important role in prognosis. Higher expression of PCNA indicates high proliferation rate and aggressiveness of tumour.

Cytolological and histopathological findings in all

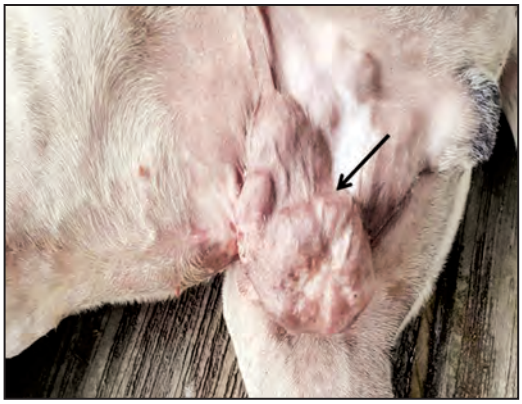

Fig. 1. Dog: Multi-nodular tumourous growths in prepucial region.

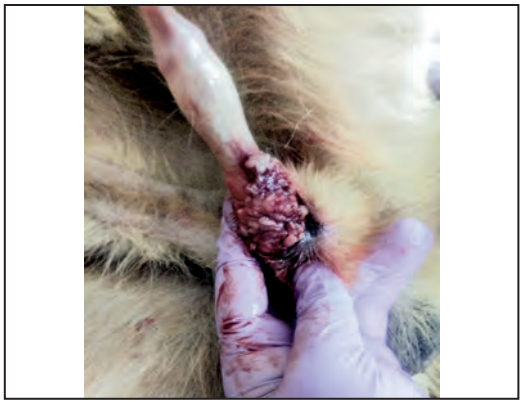

Fig. 2. Dog: Cauliflower-like growths at the base of penis.

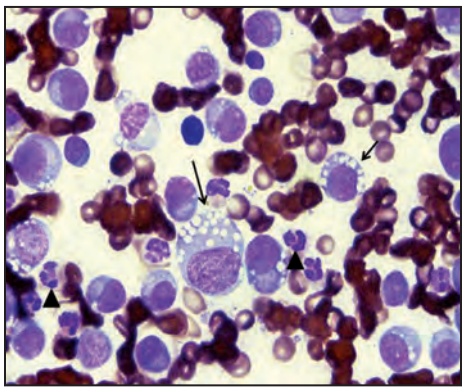

Fig. 3. TVT: Impression smear showing round cells with faint basophilic cytoplasm, characteristic cytoplasmic punctate vacuoles (arrow) and few neutrophils (arrow heads). Giemsa ×1000

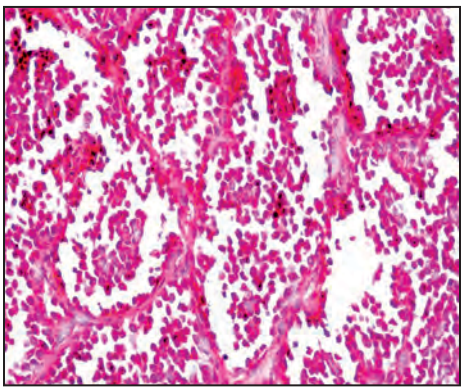

Fig. 4. TVT: Section showing round to oval shaped neoplastic cells arranged in cord-like fashion separated by thin connective tissue strands. H&E × 200

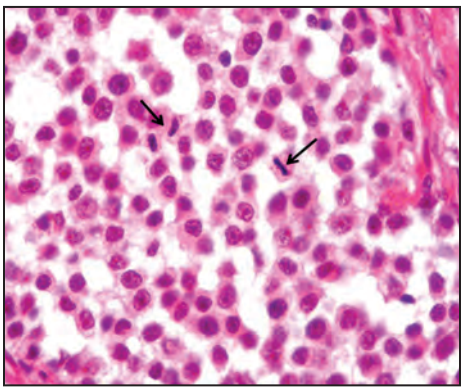

Fig. 5. TVT: Section showing neoplastic cells with large round to oval vesicular nuclei, foamy eosinophilic cytoplasm and mitotic figures (arrows). H&E×1000

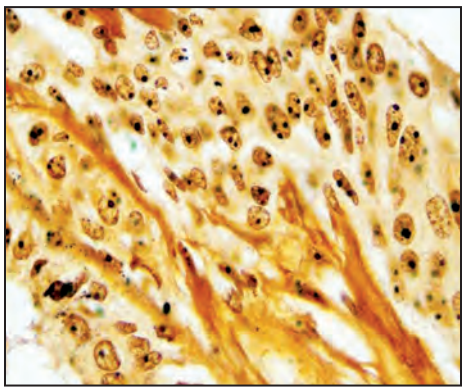

Fig. 6. TVT: Black coloured AgNOR dots in the nucleoplasm of neoplastic cells of transmissible venereal tumour. AgNOR ×1000

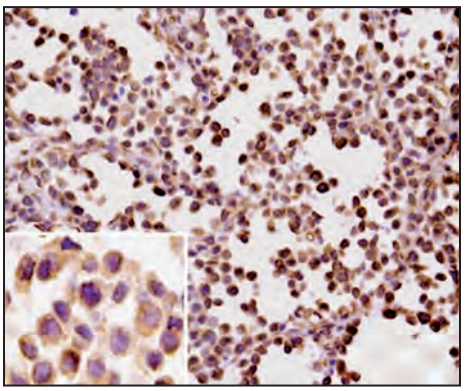

Fig. 7. TVT: Intracytoplasmic immunoreactivity for vimentin in neoplastic cells of transmissible venereal tumour and enlarged view in inset. IHC × Gill's Haematoxylin × 400

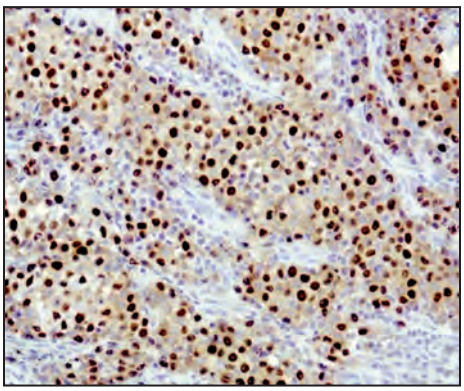

Fig. 8. TVT: Proliferating neoplastic cells of transmissible venereal tumour showing moderate to strong intranuclear immunoreactivity for PCNA. IHC × Gill's Haematoxylin × 400

the cases in the present study were similar to that described by earlier workers (Gupta and Sood, 2012; Meuten, 2017). Mitotic index is an indirect measure of proliferation of cells based on the quantification of mitotic figures. It has been demonstrated to be a strong predictor of outcome for several human and canine cancers (Romansik *et al.*, 2007). Although the TVT cases were benign in nature but MI was index high. In earlier work by Araujo *et al.* (2012) also reported high MI in TVT cases, which indicated the proliferating nature of the TVT cells. AgNOR count in TVT cases ranged from 2.01 to 3.08/nucleus which is more or less similar to that observed by Palanivelu *et al.* (2013) who reported AgNOR count of 3.38 /nucleus in TVT. MI and AgNOR count can be used as good indicator of transformation of tumours towards the malignancy (Kumar *et al.*, 2010).

Cytokeratins such as PCKs are the intermediate filament proteins which are present with in the cytoplasm of all epithelial cells and their tumours. Vimentin is an intermediate filament protein that is present in cytoskeleton of mesenchymal cells (Painter *et al.*, 2010). So, vimentin expression is primarily used to distinguish

between epithelial and mesenchymal tumours (Meuten, 2017). All the cases showed positive reaction for vimentin and negative reaction for PCK. Similarly, Gupta and Sood (2012) and Araujo *et al.* (2012) reported the vimentin positive immunoreactivity in TVT cells. The findings confirmed the mesenchymal origin of TVT.

PCNA is a non-histone nuclear protein present in the nucleoplasm of continually cycling cells throughout the cell cycle. The level of PCNA increases rapidly from late G_1 phase, most abundant during S phase and declines to an undetectable level in G_2 and M phase of cell cycle. Therefore, its rate of synthesis can be correlated directly with proliferation rate of cells (Morris and Mathews, 1989; Mukaratirwa, 2005). Moderate to strong nuclear reactivity of PCNA was observed in all the TVT cases which are in accordance with the findings of Gupta and Sood (2012) and Lokesh *et al.* (2014) who reported the strong nuclear immunoreactivity for PCNA in TVT cells. The PCNA index (40.0%) was high in one case along with high mitotic index (3.5/10hpf) and AgNOR count (3.08/nucleus) which indicated its proliferation rate of neoplastic cells and aggressiveness of tumour. It may be concluded that apart from routine cytological and histopathological examination; estimation of mitotic index, AgNOR count and immunohistochemical expression of tumour markers proves important adjunct tools for diagnosis, determination of the biological behaviour and origin of tumours.

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