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Immunohistochemical diagnosis of skin tumours in dogs

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Abstract
The present study was undertaken to evaluate the expression of potential tumour biomarkers as diagnostic and prognostic indicators in different cutaneous neoplasms. Immunocytochemical studies on *C Kit* gene in mast cell tumours showed strong positive expression of *C Kit.* Immunohistochemical investigation showed expression of cytokeratin in all the epithelial tumours and vimentin in all mesenchymal tumours studied. Among the epithelial tumours, squamous cell carcinoma showed positive expression for the marker Epithelial Membrane Antigen (EMA) in contrary to basal cell carcinoma which showed no expression to EMA. The mesenchymal tumour haemangiosarcoma showed positive expression for CD31. Among the round cell tumours, mast cell tumour showed positive expression to *C Kit,* B cell lymphoma showed positive expression to CD79a and T cell lymphoma showed positive expression to CD3. Melanoma tumours showed positive expression to both Melan A and S100.

Keywords: dogs, cutaneous tumours, skin tumours, immunocytochemistry, immuohistochemistry, tumour markers, cytokeratin, vimentin, *C Kit*, CD3, CD79a, CD31

Introduction
Cancer is the leading cause of death in dogs aged over 10 yrs, with 50 percent of older dogs developing the disease and approximately, one in four dogs eventually dying of cancer (Vail and MacEwen, 2000; Adams *et al*. 2010; Dobson, 2013) [1-3]. The critical period for the onset of neoplasia in dogs is 6 to 10 yrs (Merlo *et al*., 2008; Butler *et al.*, 2013) [4, 5]. In canines, the highest predisposition to tumours occurs in the sixth year of age and with every subsequent year the risk increases (Morris and Dobson, 2001) [6]. Skin tumours are the most common tumours in dogs (Murphy, 2006; Chikweto *et al*., 2011) [7, 8] accounting for approximately 30 percent of all diagnosed tumours (MacDonald *et al*., 2008) [9]. The incidence of cutaneous tumours in dogs is estimated to be 728 cases every year per 100, 000 dogs (Kaldrymidou *et al*., 2002) [10].

Cutaneous masses are relatively easy to be diagnosed in early stages, fortunately, by their overt manifestation on skin. However, identifying the type of tumour and its clinical behaviour seems to be unpredictable. Though cytology and histopathology are complementary diagnostic procedures and gold standards in identifying tumours, immunohistochemical studies and molecular studies would facilitate confirmatory diagnosis in undifferentiated tumours with inconclusive details in cytology and histopathology.

The advent of diagnostic immunohistochemistry using different biomarkers has made a great impact on oncology and has been referred fondly as brown revolution and magic markers (Chandrashekaraiah *et al.*, 2011) [11]. Tumour biomarkers can be used as diagnostic marker, prognostic marker, predictive marker and pharmacodynamic marker based on the need arising for individual cases. Mukaratirwa *et al.* (2005) [12] suggested immunohistochemical investigation using tumour markers to study the nature and behaviour of tumours such as to discriminate the tumours between benign to malignant, grade the tumours according to aggressiveness and metastatic risk, and to predict the prognosis, disease-free survival rates and overall survival rates after surgical excision or therapy of the tumour. Hence, the present study was undertaken to evaluate the expression of potential tumour biomarkers as diagnostic and prognostic indicators in spontaneously occurring cutaneous neoplasms in dogs.

Materials and Methods
The present study was conducted on the skin tumour suspected cases presented to the Small Animal Surgery-Out Patient ward and Small Animal Operation theatre - Surgery, of Madras

Veterinary College Teaching Hospital (MVCTH), Chennai during the period 2016-2018. A total of 175 cases were diagnosed with skin tumours based on gross, cytological and histopathological examination. Immunohistochemical investigation was carried out in representative cases of each tumour type diagnosed to study the expression of various tumour biomarkers.

The primary monoclonal antibodies to Proliferating cell nuclear antigen (PCNA), Ki67, p53 (oncogene), B-cell lymphoma 2 (Bcl2) (oncoprotein), Cytokeratin, Vimentin, EMA, Desmin, E Cadherin, *C Kit*, CD 79a, CD 3, Melan A and S100 and secondary antibody (Poly Excel HRP (Horseradish peroxidase)/DAB (3, 3'–diaminobenzidine) detection system) were purchased from VKAN CARE, USA. (M/s. PathnSitu, Biotechnologies Pvt. Ltd., USA).

Immunocytochemistry

Air dried direct smears of 12 cases of MCTs taken on APES/Poly L-Lysin precoated slides were fixed immediately or post fixed just prior to staining in 95 percent ethanol or cold acetone for 30 min, wrapped in aluminium foil and preserved at -20ºC. Immunocytochemical staining were conducted using commercial antibodies to the markers.

Immunohistochemistry

Immunostaining for PCNA, p53, Cytokeratin, Vimentin, Desmin, and C kit, CD3, CD 79a, Melan - A and S 100 markers were done on formalin fixed paraffin embedded tissues using HRP polymer and the staining method was carried out as per the standard procedure recommended by the manufacturer. 3-4 µm thick paraffin sections collected on slides were coated with poly-L lysine and dried at 56ºC overnight. They were then deparaffinised in xylene (I, II and III) for 15 minutes (5 minutes each) and rehydrated through isopropanol (I and II, 2 minutes each). Slides were washed in running tap water for 10 minutes and then in distilled waterand placed in microwaveable plastic jars filled with 200 mL of antigen retrieval solution and heated in microwave for 15-20 minutes at 120ºC.

The slides were taken from the oven, placed in room temperature for 20 minutes, washed in running tap water for 5 minutes and washed with phosphate buffered saline (3 minutes x 3). The slides were over layed with 3% hydrogen peroxide block (H_2O_2) in distilled water for 5 minutes, washed with phosphate buffered saline (5 minutes x 3) and treated with power (protein) block solution for 5 minutes. Primary antibody was added to the slides and the slides were incubated for 1-2 hours, washed with phosphate buffered saline (5 minutes x 3) and further incubated with poly HRP for 30 minutes (in dark room), washed with phosphate buffered saline (5 minutes x 3) and treated with Pathnsithu/DAB (Stunn chromogen-1 drop) and substrate buffer (Stunn buffer-0.5 ml) for 30 seconds -2 minutes. The slides were finally washed in running tap water, counter stained with haematoxylin for 3 minutes, washed with tap water for 3-5 minutes and then dried and mounted with DPX.

Results and Discussion

Immunocytochemical and immunohistochemical study done on various types of cutaneous tumours were evaluated for positive and negative expression. Positive expression was further graded based on the percentage of cells showing positive expression, as mild (less than 20% of cells showing expression), moderate (20-50% of the cells showing expression) and strong (more than 50% of the cells showing expression).

Immunocytochemical findings

Air dried direct smears of mast cell tumours, obtained by Fine Needle Aspiration Cytology and taken in APES/Poly L-Lysin precoated slides showed focal to moderate expression of *C Kit* (n = 9) (Plate 1, 2). Strong and diffuse expression of *C Kit* staining was observed in three cases which was suggestive of high grade MCTs. Earlier, Sailasuta *et al*. (2014) [13] also stated increased opportunity to observe a higher *C Kit* staining pattern in high-grade MCT by immunocytochemistry. Thus immunocytochemistry can be an effective, safe, non-invasive method in routine clinical settings to diagnose as well as to predict the prognosis of MCTs based on the intensity of *C Kit* expression.

Immunohistochemical findings

Proliferating cell nuclear antigen (PCNA)

Pardee (1989) [14] stated that cell proliferation reflected the number of cells in the cell cycle (growth fraction) and the rate at which cells progressed through the cell cycle (proliferation rate). The immunohistochemical biomarker PCNA was employed in the present study to understand the proliferation rate of the tumour cells which in turn would indicate the aggressiveness or recurrence potential of the tumour cells. Immunohistochemical staining revealed strong and diffuse positive expression of PCNA in papilloma (Plate 3), squamous cell carcinoma (Plate 4) and sebaceous gland adenocarcinomas. Mast cell tumours also showed strong positive expression for PCNA indicating the proliferating ability of these tumours.

Maiolino *et al*. (1995) [15] opined that PCNA index was of great value in separating histologically similar canine tumours like cutaneous squamous cell carcinoma and basal cell carcinoma. Abadie *et al*. (1999) [16] observed that disease free survival and overall survival time was longer in MCTs with a low PCNA index. Hung *et al*. (2000) [17] suggested PCNA index as a useful tool in grading MCTs according to their degree of aggression. Ishikawa *et al*. (2006) [18] stated that positive expression of PCNA is an indication of proliferative ability of the tumours, thus high PCNA expression is an indicator of more aggressive growth and recurrence.

Tumour suppressor Gene p53

Lopes *et al.* (2010) [19] stated that p53 also known as "the guardian of the genome" was located in chromosome 17 in human and in chromosome 5 in dogs. They added that it was the most common target of genetic changes caused by tumours in humans and other animals. In our study, weak to moderate positive expression of p53 gene was seen in squamous cell carcinoma, trichoblastoma, sweat gland adenocarcinoma, mast cell tumour (Plate 5), histiocytoma (Plate 6) and haemangiopericytoma. Positive p53 gene expression was seen as a brown coloured staining within the nucleus.

The naturally occurring p53 gene in healthy skin may not be expressed in immunohistochemical staining due to its short half-life of less than an hour.But the expression of p53 in neoplastic cells is attributed to cancer associated forms of p53 which have long half-life to be detectable in immunohistochemistry and it also denotes the aggressiveness of the tumour (Sirvent *et al*., 2004) [20]. Jaffe *et al.* (2000) [21] studied the immunohistochemical over expression of p53

protein in canine tumours and observed that over expression of p53 in mast cell tumours was found to be of diagnostic significance in grading of mast cell tumours in dogs.
However, the presence of moderate to weak expression to p53 as seen in the present study even in malignant tumours confirmed by histopathological examination could be due to mutation of the type deletion, wherein the gene would have been lost for which mutant p53 gene fail to be expressed. This was earlier reported by Montiani-Ferreira *et al.* (2008) [22] that the negativity for p53 staining is due to deletion of the p53 gene resulting in the absence of p53 protein in neoplastic cells.

Panel of immunohistochemical markers
In addition to the proliferation marker PCNA and the tumour suppressor gene p53, immunohistochemical study for various tumours was done employing markers for detecting the origin of tumour like cytokeratin, vimentin and a chosen set of specific markers to identify the tumour like C kit for Mast cell tumours, CD3 and CD79a for lymphoma and Melan A, S 100 for melanocytic neoplasms. The panel of markers for the different types of tumour and the results are presented in Table 1-4.

Immunohistochemical panel for epithelial tumours
Cytokeratin and Vimentin
All the epithelial tumours showed positive expression to the epithelial marker cytokeratin and negative expression for vimentin suggesting the epithelial origin of the tumours. This was in accordance with Toniti *et al*. (2010) [23] who reported cytokeratins as fundamental markers of epithelial differentiation. Positive expression to cytokeratin was seen as brown coloured staining in the cytoplasm. The expressions were observed in varying intensities among the various epithelial tumours as moderate and strong positive expression. Papilloma and perianal gland adenocarcinomas showed strong positive expression of cytokeratin (Plate 7, 8). Squamous cell carcinomas, trichoblastomas, sweat gland adenomas, sweat gland adenocarcinomas, and ceruminous gland adenomas showed moderate expression of cytokeratin.

Epithelial membrane antigen (EMA)
To differentiate two cases of highly anaplastic squamous cell carcinomas from trichoblastoma, both of which showed positive expression of cytokeratin due to epithelial origin, Epithelial Membrane Antigen (EMA) was employed. Strong positive reaction for EMA was observed which confirmed the tumour as squamous cell carcinoma in a case. Another case showed negative expression for EMA based on which it was diagnosed as basal cell carcinoma. The immunohistochemical findings were in agreement with the findings of Ramezani *et al*. (2016) [24]who stated that squamous cell carcinomas had considerable immunoreactivity to Epithelial Membrane Antigen compared to basal cell carcinoma.

Immunohistochemical panel for mesenchymal tumours
Cytokeratin and Vimentin
A total of 40 cases of mesenchymal tumours were diagnosed by histopathological examination which included 18 cases of lipoma, 9 cases of liposarcoma, seven cases of fibrosarcomas and two cases each of haemangiosarcoma, haemangiopericytoma and fibroma.
Among the 40 cases, paraffin embedded tissue sections of seven cases of fibrosarcomas, two cases each of haemangiosarcoma, haemangiopericytoma, fibroma, lipoma and liposarcoma were stained with immunohistochemical markers, cytokeratin and vimentin to identify the origin of the tumours. The positive reaction to vimentin was seen as brown coloured staining in the cytoplasm. Strong expression of vimentin was seen in fibroma, fibrosarcoma (Plate 9) and mild to moderate expression was observed in lipoma, liposarcoma, haemangiosarcoma and haemangiopericytoma. Further, all the tumours showed no positivity for cytokeratin which confirmed the mesenchymal origin of the tumours.

CD31
Two cases of mesenchymal origin had histopathologic features of haemangiosarcoma. Immunohistochemistry was carried out employing CD31 marker to have a confirmatory diagnosis. Neoplastic cells showed mild to moderate expression of CD31 which confirmed the tumours as haemangiosarcoma.Earlier, Tsuji *et al.* (2013) [25] have also diagnosed a case of canine haemagiosarcoma based on positive expression for CD31. Maharani *et al*. (2018) [26] stated the negative correlation between CD31 and cellular atypia in canine hemangiosarcoma.

Immunohistochemical panel for round cell tumours
Immunohistochemical markers were used in selected cases of closely resembling round cell tumours to make a confirmatory diagnosis in addition to employment of special stains like toluidine blue for mast cell tumours. The candidates of choice in the panel for immuohistochemical diagnosis were *C Kit* for mast cell tumours, E Cadherin for histiocytoma, CD3 for T cell lymphoma and CD79a for B cell Lymphoma.

Cytokeratin and Vimentin
Mast cell tumours in the present study showed strong positive reaction to vimentin (Plate 10) and no positive expression for cytokeratin. The positive expression to vimentin was seen as dark brown staining in the cytoplasm. TVT (n = 3) cells also showed positive expression to vimentin. However, histiocytoma and lymphoma cells showed either mild expression or absence of expression.

C Kit
The positive reaction to *C Kit* was observed in all the MCTs studied for *C Kit* expression. Tumours showing both moderate expression (Plate 11) and strong expression (Plate 12) were encountered. The positive reaction was seen as diffuse brown staining of the cytoplasm. In three cases, strong diffuse cytoplasmic expression was observed which indicated the aggressiveness of the tumour. Morini *et al.* (2004) [27] observed strong positivity of CD 117 in normal mast cells of the skin with two staining patterns namely, membrane associated and diffuse cytoplasmic staining. They observed an additional CD117 staining pattern in immunohistochemical staining of MCT tumours namely the paranuclear pattern which was not expressed by the resting mast cells of the skin. Preziosi *et al.* (2004) [28] have stated that diffuse pattern was seen in MCT grade I and paranuclear pattern in Grade III. Kiupel *et al.* (2004) [29] also stated that a more aggressive biologic behaviour of canine cutaneous MCTs was associated with the increase in cytoplasmic staining for *C Kit* as the animals with diffuse cytoplasmic staining had reduced survival time and recurrence post-surgical excision of MCTs. The immunohistochemical findings thus favoured the employment of *C Kit* as a useful immunohistochemical

marker for identification of mast cell tumours among the immunohistochemistry panel for round cell tumours.

CD3 and CD79a

Two cases of histopathologically confirmed cutaneous lymphomas in our study were subjected to immunohistochemical investigation to identify the type of lymphoma by employing CD3, CD79a, Vimentin and C Kit. Moderate expression of CD 79a marker was seen in a case suggesting it as B cell Lymphoma. CD79a expression was seen as a brown, moderate cytoplasmic expression in a case of B cell lymphoma (Plate 13). The differentiation between cutaneous B cell Lymphoma and plasma cell tumour (n = 3) was further established by observing the eccentric nucleus and perinuclear halo in histopathologic examination to support the diagnosis, as CD79a is expressed by both B cell lymphoma and plasma cell tumour as reported earlier by Ginn *et al.* (2007) [30].

Strong positive expression to CD3 marker was seen in another case suggesting it as a case of T cell Lymphoma. Strong positive CD3 expression was seen in a case of T cell lymphoma as a very dark brown cytoplasmic and membrane staining of neoplastic lymphocytes (Plate 14). This was similar to the findings of Fournel-Fleury *et al.* (2002) [31] who reported CD3 is a specific marker for T lymphocyte.

Immunohistochemistry has shown to be an important tool in precise disease diagnosis of lymphoid tumours (Dobson *et al.*, 2002) [32], making it possible to classify either T or B-cell Lymphoma and also the degree of neoplastic lymphoid cell maturation. The technique has been successfully applied in paraffin-included histological material, marking B-cell lymphomas with monoclonal antibody anti-mb1 (CD79a) and T-cell lymphomas with polyclonal antibody anti-CD3 (Fournel-Fleury *et al.*, 1997; 2002; Bhang *et al.*, 2006[]; Cardoso *et al.*, 2006; Arespacochaga *et al.*, 2007) [33, 31a, 34-36]. Araujo *et al.* (2012) [37] observed positive CD3 expression in all 12 cases of cutaneous T cell lymphoma and positive expression of CD79a in a single case of cutaneous B cell lymphoma in a study on total 13 cases of canine cutaneous lymphoma.

According to the literature, most canine lymphomas are type B (Teske *et al.*, 1994; Fournel-Fleury *et al.*, 1997; Arespacochaga *et al.*, 2007) [33a, 36a, 38]. But in our study, the number of cases was limited, to make a statistical interpretation, only two out of 175 cases. Of which, one case was diagnosed as T cell lymphoma and another case as B cell lymphoma by positive expression to the markers CD3 and CD79a respectively.

E-cadherin

Mild to moderate expression of E-cadherin was observed in two out of the six cases of histiocytoma subjected to immunohistochemical investigation. The inability to detect expression in the other cases could be attributed to the fact the tumour cells are in the stage of regression in such cases. Pires *et al.* (2009) [39] have reported that the intensity of the E-cadherin immunolabeling in canine cutaneous histiocytoma decreased in the presence of lymphoid infiltration and further added that the loss of E-cadherin expression might represent maturation process of the tumour cells in canine cutaneous histiocytoma.

Immunohistochemical panel for melanocytic tumours

In the present study, two cases of melanoma identified were pigmented melanomas. Immunohistochemical staining for both the cases was done with the basic markers cytokeratin and vimentin and for melanoma specific markers Melan A and S100.

Cytokeratin and Vimentin

Melanoma (n = 2) showed no positive expression to cytokeratinbut showed strong expression for vimentin.

Melan – A and S100

Ramos-Vara *et al.* (2000) [40] have stated Melan A as a specific and sensitive marker of melanomas while (Choi and Kusewitt, 2003 [41] have reported S100 as a sensitive marker for the colourless, amelanotic melanomas. Hence, both the markers Melan A and S100 were employed in the present study to observe the sensitivity of these markers in identifying melanoma. Melanoma (n = 2) showed strong expression for both the melanoma specific markers S100 and Melan A. The positive expression was seen as brown coloured staining in the cytoplasm. This is in close agreement to the findings of Wilkerson *et al.* (2003) [42] who reported negative expression of cytokeratin, and positive expression for S100 and Melan A in melanocytic neoplasms.

Table 1: Immunohistochemical panel for epithelial tumours

Tumour	Cytokeratin	Vimentin	EMA
Papilloma (n = 6)	+	-	ND
SQCC (n = 6)	+	-	+ (2/2)
Basal cell carcinoma (n = 6)	+	-	- (2/2)
Perianal gland tumours (n = 6)	+	-	ND
Sweat gland tumours (n = 6)	+	-	ND
Sebaceous gland tumours (n = 6)	+	-	ND
Ceruminous gland tumours (n = 6)	+	-	ND

***** ND – Not done

Table 2: Immunohistochemical panel for mesenchymal tumours

Tumour	Cytokeratin	Vimentin	Desmin	CD31
Lipoma (n = 6)	-	+	ND	ND
Liposarcoma (n = 6)	-	+	ND	ND
Fibroma (n = 2)	-	+	-	ND
Fibrosarcoma (n = 6)	-	+	-	ND
Haemangiosarcoma (n = 2)	-	+	-	+
Haemangiopericytoma (n = 2)	-	+	-	-

***** ND – Not done

Table 3: Immunohistochemical panel for round cell tumours

Tumour	Cytokeratin	Vimentin	C Kit	E Cadherin	CD79a	CD3
MCT (n = 22)	-	+	+	-	-	-
PCT (n = 3)	-	+	-	-	+	-
Lymphoma (n = 2)	-	±	-	-	+	+
Histiocytoma (n = 6)	-	±	-	+ (2/6)	-	-
TVT (n = 3)	-	+	-	-	-	-

Table 4: Immunohistochemical panel for melanocytic tumours

Tumour	Cytokeratin	Vimentin	MelanA	S100
Melanoma (n = 2)	-	+	+	+

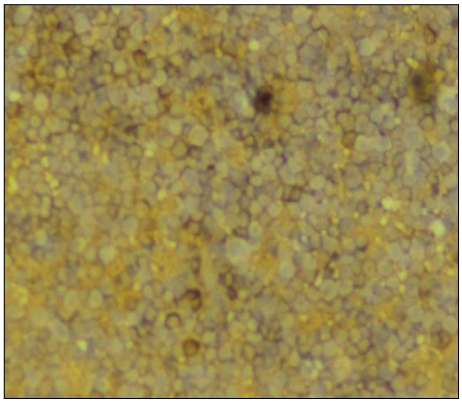

Plate 1: ICC – *C Kit* - MCT- Moderate expression x 400

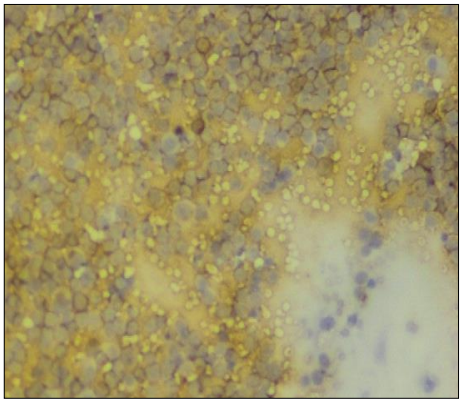

Plate 2: ICC – *C Kit* - MCT- Moderate expression x 400

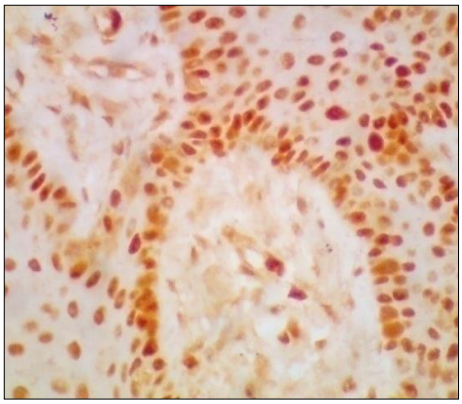

Plate 3: IHC - PCNA - Papilloma – Strong expression x 200

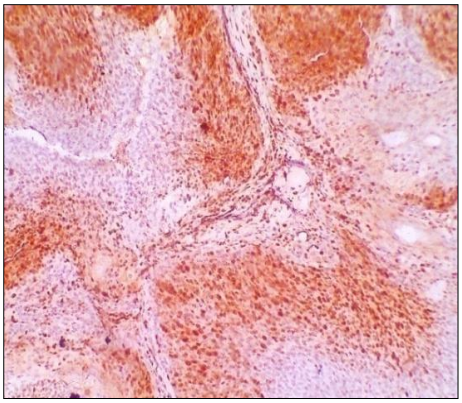

Plate 4: IHC – PCNA - Squamous cell carcinoma – Strong expression x 100

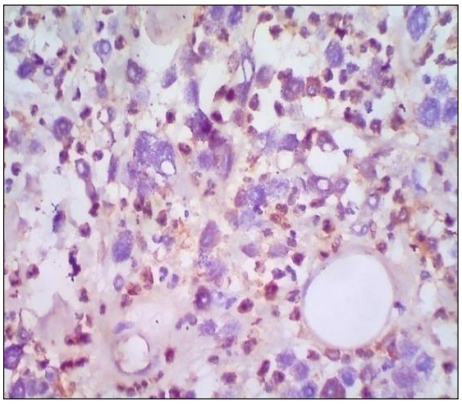

Plate 5: IHC – p53 - Mast cell tumour – Moderate expression x 200

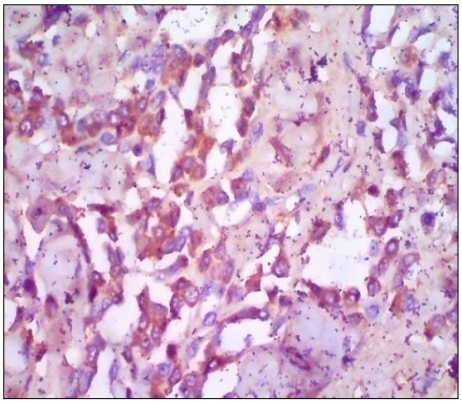

Plate 6: IHC – p53 - Histiocytoma - Moderate reaction x 400

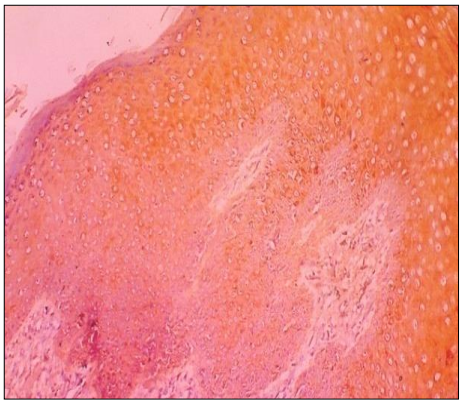

Plate 7: IHC - Cytokeratin - Papilloma – Strong expression x 40

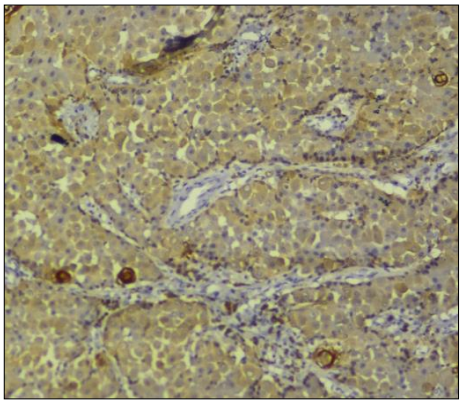

Plate 8: IHC – Cytokeratin - Perianal gland adenocarcinoma – Strong positive expression x 400

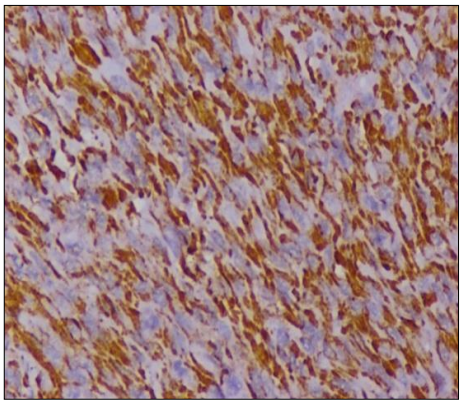

Plate 9: IHC – Vimentin - Fibrosarcoma – Strong expression x 100

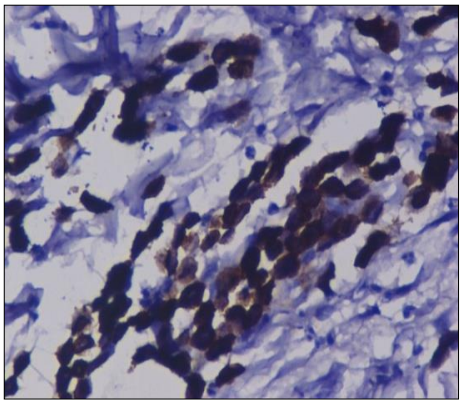

Plate 10: IHC - Vimentin – Mast cell tumour - Strong expression x 400

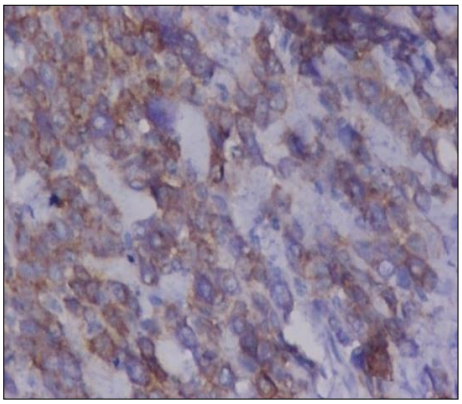

Plate 11: IHC – C Kit - Mast cell tumour – Moderate expression x 400

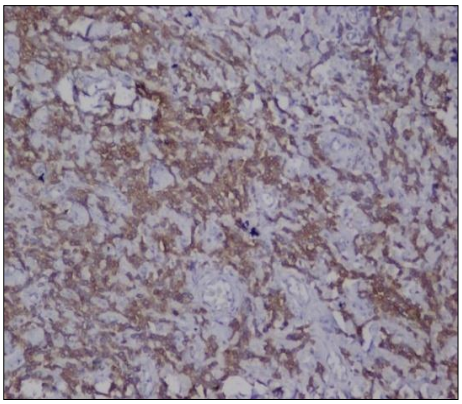

Plate 12: IHC - C Kit - Mast cell tumour - Strong expression x 100

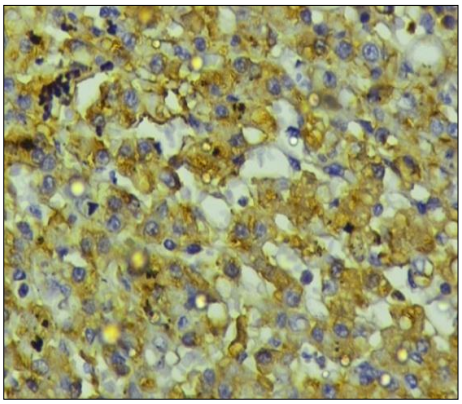

Plate 13: IHC - CD79a - B cell Lymphoma - Moderate expression x 400

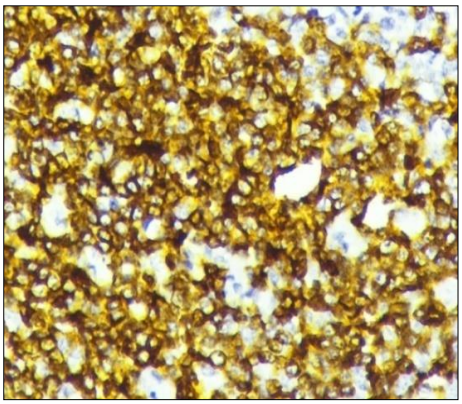

Plate 14: IHC - CD3 - T cell Lymphoma -Strong expression x 400

Conclusion

Immunohistochemical investigation requires skill and expertise and is a bit expensive to be employed for all tumour suspected cases. However, they serve as a guiding tool in the diagnosis of poorly differentiated, anaplastic cases where cytological and histopathological diagnosis is inconclusive. Selective immunohistochemical markers based on the results of histopathology can be employed which will be of great value in augmenting diagnosis of tumours in early stages, in understanding the malignancy features and recurrence rate and to ascertain the prognosis in clinical settings.

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Conflicts of interest

There is no conflict of interest.

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