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Immunohistochemical Evalution of Endoglin (CD 105) as Angiogenic Endothelial Marker of Squamous Cell Carcinoma in Dogs

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ABSTRACT

Back ground: Angiogenesis is the process of using existent vascular bed to form new blood vessels. Neoangiogenesis is one of the hallmarks of cancer for tumour progression and metastasis. Microvessel density (MVD) is a powerful prognostic tool in oncology for assessing the tumour vasculature. Endoglin (CD 105) is a specific marker for activated endothelium. The present research intends to investigate the prognostic and metastatic potential of Endoglin (CD 105) and immunohistochemical expression in canine squamous cell carcinoma (SCC).

Method: Totally, 15 SCC were collected from the Veterinary Clinical Complex, Tirunelveli during a one year period. The tumours were histopathologically identified as different grades. Immunohistochemically analysed with Endoglin (CD 105) and Peritumoural microvessel density and intratumourl microvessel density (PTMVD and ITMVD) were calculated using the “hot spot method”.

Result: All the cases were positive for CD 105 and were strongly stained with both peritumoural and intratumoural blood vessels. Among the different types of SCC, Grade III had the highest values of microvessel density. The study concluded that different grades have different MVD values within SCC cases, thereby reflecting the behaviour of neoplasms directly. Endoglin (CD 105) is one of the best endothelial markers to determine MVD because it aids to assess the prognostic and metastatic potential of tumours and it plays a significant role in the estimation of overall survival period after surgery.

Key words: Dog, Endoglin (CD 105), Immunohistochemistry, Squamous cell carcinoma.

INTRODUCTION

Squamous cell carcinoma (SCC) is highly invasive and it originates from different types of epithelial surfaces. Oral and eye SCCs are more dangerous and these are diseases recognised from genetically altered cells, which cause uncontrolled growth of damaged cells (Meuten, 2017). Angiogenesis is the process of using existent vascular bed to form new blood vessels. Neoangiogenesis is one of the hallmarks of cancer for tumour progression and metastasis. Angiogenic activity could be detected by measuring various features of tumourous blood vessels. Microvessel density (MVD) is a powerful prognostic tool in oncology for assessing the tumour vasculature. MVD directly reflects the angiogenesis (Preziosi *et al.* 2004). A number of studies proved that many antibodies are used for highlighting the blood vessels using the immunohistochemical technique (IHC) Hasan *et al.* (2002). Only a proportion of lymphatic endothelium and capillaries, which are the primary endothelial markers in feline and canine neoplasms, is detected by von Willebrand factor (vWf) Weinder *et al.* (1991). The most specific endothelial marker is CD 31-Platelet endothelial cell adhesion molecule-1. CD 31 is the pan endothelial marker and its takes all blood vessels (Maiolino *et al.,* 2000; Kumar *et al.,* 2014). Although CD 34 has expressed some stromal cells, it is a good alternative antigen Li *et al.* (1994). Endothelium is highly heterogeneous in nature (Carthy *et al.* 1991) and with a high proliferative rate, it is known as activated endothelium (Fonsatti *et al.* 2000). Endoglin (CD 105) is a specific marker for activated endothelium (Tae *et al.* 2000). Endoglin expression is a better prognostic indicator than CD 31 and vWf (Legan, 2005; Duff *et al.,* 2003; Rao *et al.,* 2010). CD 105 is a glycoprotein of type 1 membrane, which constitutes the receptor complex of TGF-β on the cell surface. Its principal location is on human chromosome 9 (Maharudrappa *et al.* 2014). It might

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play a definitive role in the formation of tumours (Fernandez *et al.* 1993). The expression of CD105 is correlated to tumour size, lymph node metastasis and prognosis studied in many human cancers (Rao *et al.* 2010). CD105 expression intensity has also been correlated to a poor prognosis and the most powerful marker of neo vascularisation (Azadeh and Ashraf, 2014). After surgical excision of tumours, it is necessary to assess the prognostic and metastatic potential. Animal oncology still has a gap between diagnosis and prognosis and the behaviours of neoplasm need to be found. Only few researchers have investigated the CD 105 expression in canine tumours for prognostic and metastatic purposes. The present study intends to use CD 105 for evaluating MVD as a predictor of survival, metastatic propensity, rate of cellular proliferation and histological features and grade in SCC.

MATERIALS AND METHODS

Fifteen SCC tissue samples were collected through excisional biopsy of dogs brought to Small Animal Clinic at Veterinary Clinical Complex, Veterinary College and Research Institute, Tirunelveli from April 2021 to April 2022. Case details, such as breed, age, sex, location, size, shape, weight and gross appearance of skin tumours were documented. Fine needle aspiration cytology was performed following the standard procedure. Smears prepared from tumours were stained with Leishman-Giemsa stain. Surgical tissue samples were routinely processed for histological examination after they were fixed in 10% neutral buffered formalin. Paraffin embedded tissues were stained with haematoxylin and eosin. Further Parafin-embeded tissues were cut to 4 µm thickness (Kim *et al.* 2013) for immunohistochemistry.

Immunohistochemistry

Protocol was followed for performing immunohistochemical staining in 15 surgically excised tumour samples using the super sensitive labelled HRP polymer method. Briefly, sections of 4 µm thick paraffin embedded tissue samples were collected on slides coated with poly- L- lysine dried at 56°C for 3-5 h. Xylene was used to deparaffinise, grades of alcohol were used to rehydrate and 0.01M PBS pH 7.4 was used to wash the paraffin sections. Hydrogen peroxide (0.3%) was used for 30 min in methanol to quench endogenous peroxidase. After washing twice in PBS, 1M Tris EDTA buffer (pH-9) was used for 15 min to retrieve antigen in a pressure cooker. The sections were treated with protein block (Goat serum) for 30 min, followed by washes in PBS. The sections were incubated using the primary antibody CD 105 (Ready to use) clone (EP274) from Pathnsitu (USA). Only diluent was applied for negative control and sections were incubated for 1h in a humidified chamber. The slides were sequentially treated with super enhancer and super sensitive horse radish peroxidase (HRP) for 30 min after being rinsed twice with PBS. Diaminobenzidine (DAB) was used to develop a dark brown reaction product, counter stained with haematoxylin and washed with tap water, air dried, cleared with xylene and mounted with DPX mountant.

Microvessel density quantification

Endoglin (CD 105) expression was evaluated through quantitative analysis at high power magnification as per recent consensus (Vermeulen *et al.* 2002). Immunohistochemically stained slides were analysed for MVD count and CD 105 positive was confirmed by the staining of brown coloured endothelial cells found in the vessel wall. MVD was determined using the hotspot method after its positivity was confirmed using Olympus CX-41 microscope with an Image-Pro-plus version 5.1. The slides were initially examined through a 10× magnification. The areas with the highest quantity of blood vessels stained with CD 105 positive (hotspots) were discovered and chosen for further analysis.The same field was viewed under 40× magnification and 10 hotspots were selected from the peritumoural microvessel density (PTMVD) and intratumoural microvessel density (ITMVD) areas. The averages of these 10 hotspots were measured as the number of vessels per mm^2. The outcomes were expressed as the average number and the highest number of microvessels per mm^2. T-test and paired t test were used for comparing the mean values of tumours. A statistical *p* value of less than or equal to 0.05 was regarded as significant.

RESULTS AND DISCUSSION

The details of the SCC cases are furnished (Table 1). In the present study, the tumours occurred in six Non-descript, four Spitz and each two Labrador and Doberman and one Rottweiler. The affected animals' age varied from 3 years to 13.5 years. The mean age of occurrence was 8.6 years. Goldschmidt *et al.* (1998) stated that older dogs were more prone to cutaneous tumours, where in eleven cases were males and four cases were females. The tumours were located in the eyelid, nasal bridge, thorax, ventral abdomen, shoulder region, thigh region, base of the tail, upper jaw, anal region, preputial area, vulva, neck (Fig 1) and fore digit. Henry *et al.* (2005) reported that the most common tumour encounterd in digits was squamous cell carcinoma. Grossly, the masses measured 7-8 cm in diameter or irregular, round to oval in shape or cauliflower-like in appearance and reddish to grey in colour. Surface ulcerations and necrosis were observed in three cases.The cut section revealed greyish white and consistency was hard. Cytology revealed moderate to high amount of cells arranged in clusters. The neoplastic cells were polyhedral in shape with pale blue cytoplasm. Neoplastic cells at different stages of differentiation showing anisocytosis and anisokaryosis were observed. Nuclei were spherical and contained coarse chromatin. Single to multiple basophilic nucleoli were observed. Binucleate cells and tadpole cells were observed in the study that concurred with the report of with (Kumar *et*

al. (2018). In this study, histologically, tumours were divided into three categories, *viz*. Grade I, Grade II and Grade III. SCC was well differentiated in six cases in Grade I, moderately in four cases in Grade II and poorly in five cases in Grade III. Well differentiated cases (Grade I) revealed cell nests of varying size containing keratin pearls at the centre. Keratin pearls caused compression and atrophy of the surrounding neoplastic cells. The neoplastic cells were polyhedral in shape and were arranged in multiple layers (Fig 2) that contained vesicular nuclei with one or two basophilic nucleoli. Intercellular bridges were observed. Cell nests revealed hyperchromatic nuclei and vacuolar degeneration. One case showed many mitotic figures. Occasionally, atypical mitosis and neovascularisation were also observed. Moderately differentiated cases (Grade II) showed neoplastic cells, increased pleomorphism, nuclear cytoplasmic ratio, marked mitotic figures (Fig 4) and few keratin pearls (Fig 3). Grade III of poorly differentiated tumours revealed numerous single neoplastic cells with a high number of mitotic figures and scanty cytoplasm.These were in accordance with the report of Mathew *et al.* (2020). The most common histological type in the present study was the well differentiated carcinoma, followed by the moderately differentiated carcinoma, which is according to a previous finding in human oral SCC Margaritescu *et al.* (2008).

Immunohistochemistry of CD 105

Proliferating blood vessels and vascular endothelial cells were distinctly identified through Endoglin (CD105) and brown coloured staining. All cases were positive for CD 105

Table 1: Breed-age-sex wise occurrence of squamous cell carcinoma in dogs.

Breed	Age in years			Sex		Place
	0-5	6-10	11-15	Male	Female	
Non-descript	3.0	-	-	-	1	Caudal thorax
Doberman	-	9.0	-	1	-	Neck region
Non-descript	5.0	-	-	1	-	Thigh region
Rottweiller	-	8.0	-	1	-	Upper Jaw
Sptiz	-	-	12.0	-	1	Ventral abdomen
Sptiz	-	-	10.5	1	-	Preputial area
Labrador	-	-	11.0	1	-	Shoulder region
Non-descript	-	-	10.0	1	-	Anal region
Sptiz	-	-	11.0	1	-	Tail
Non-descript	-	-	12.0	-	1	Digit
Doberman	-	9.5	-	1	-	Nasal bridge
Labrador	-	-	12.0	1	-	Digits
Sptiz	-	9.0	-	-	1	Tail
Non-descript	-	-	13.5	1	-	Vulva
Non-descript	-	-	12.0	1	-	Eyelid
Total	02	04	09	11	04	

Table 2: Microvessel density count of squamous cell carcinoma.

Tumour type	Peritumoural microvessel density (PTMVD) Mean±SD	Intratumural microvessel density (ITMVD) Mean±SD	Overall survival <3 months	Overall survival <6 months
Well differentiated	26.8±4.97	13.8±0.84	Alive	Alive
Well differentiated	27.8±1.64	13.6±1.67	Alive	Alive
Well differentiated	20.4±3.05	13.8±3.27	Alive	Alive
Well differentiated	23.2±1.48	13.4±2.30	Alive	Alive
Well differentiated	28.2±1.30	16.4±4.98	Alive	Metastasis
Well differentiated	32.8±2.59	16.8±4.09	Alive	Metastasis
Moderately differentiated	29.8±5.40	15.2±1.30	Alive	Alive
Moderately differentiated	26.0±3.16	15.2±6.98	Alive	Alive
Moderately differentiated	30.0±1.58	13.6±2.40	Death	-
Moderately differentiated	28.6±4.15	14.4±2.70	Alive	Alive
Poorly differentiated	32.4±2.07	14.0±3.16	Alive	Alive
Poorly differentiated	32.8±1.64	16.4±1.14	Death	-
Poorly differentiated	33.2±1.92	17.0±1.58	Death	-
Poorly differentiated	31.6±2.60	14.6±1.14	Alive	Death
Poorly differentiated	31.6±1.67	15.2±1.92	Alive	Death

and moderate staining was recorded in the invasion of skeletal muscles and keratin pearls. CD 105 strongly stained both peritumoural and intratumoural blood vessels. The tumour blood vessels were convoluted, lacked a distinct lumen, had extensive gaps between endothelial cells and were taken for intense staining (Fig 5). In well-differentiated SCC vascular network were multiple vascular bed, aberrant morphology, tortuous and clear lumen highlighted by CD 105. Numerous blood vessels (smaller than 15 µm diameter) and a high amount of individual endothelial cells were seen in poorly differentiated tumours. It stromal regions near carcinomatous proliferations below the dermis and between the lobules (Fig 6) had the highest MVD. Moderately differentiated SCC had intratumoural and peritumoural areas blood vessels that were less complex with few vascular loops and high lumen width. In all the cases, peritumoural areas had more blood vessels and numerous endothelial cells. These findings were compatible with previous investigations Tadbir *et al.* (2014) and Margaritescu *et al.* (2008).

The mean MVD of SCC is furnished - (Table 2). Several researchers have identified MVD by examining pan-endothelial markers, such as VEGF-A, CD34 and CD31; however, these markers have low sensitivity and specificity and the results are there for inconclusive. On the other hand, CD 105 demonstrates its high specificity through interactions with proliferating (angiogenic) endothelial cells in MVD. Endoglin (CD 105), one of the most specific endothelial markers, aids in assessing tumour progression because it plays a significant role in neoangiogenesis Margaritescu *et al.* (2008). MVD using CD 105 is considered as a useful prognostic indicator in the treatment of several human cancers. These findings are in agreement with the study of Kuriakose *et al.* (1992). In this research, all cases of SCC were evaluated immunohistochemically and histopathologically. All cases were positively expressed by CD 105 antibodies and high intensity was observed in poorly differentiated cases. These findings show that CD 105 might be vital for developing tumours and it is more abundant in tumour tissues. The marker was more expressed in invasive tumours than in benign ones. This finding supports the previous findings that endoglin reacts only with tumour-derived angiogenic endothelial cells Tadbir *et al.* (2014). MVD in all histological grades of SCC was the highest at peritumoural region, followed by intratumoural region. There was a significant different between PTMVD and ITMVD. The results were significant at $p<05$. There was no significant difference between the mean of well differentiated cases and moderately differentiated cases. But there was significant difference between well differentiated cases and poorly differentiated cases. This finding concurred with previous observation that CD105 expression decreases as it moves away from invasive front of the tumour, as it aids invasion into the dermis Kyzas *et al.* (2006). No significant association of MVD was found with age, sex, size and location. This finding concurred with the report of Margaritescu *et al.* (2008). Two cases showed higher ITMVD and PTMVD among the well differentiated SCC cases were 28.2±1.30, 32.8±2.59 and 16.4±4.98, 16.8±4.09, respectively. In these cases, metastasis occurred after six months. The case with the highest MVD (PTMVD 30.0±1.58 and ITMVD 13.6±2.40) in the moderately differentiated group died within three months of post survival analysis. This result concurred with

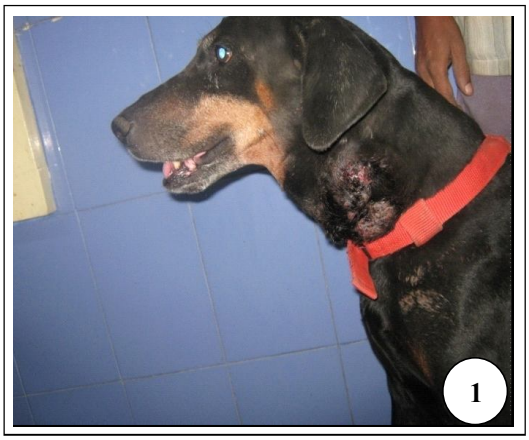

Fig 1: SCC-Doberman-Male-Ulcerated mass-Neck region.

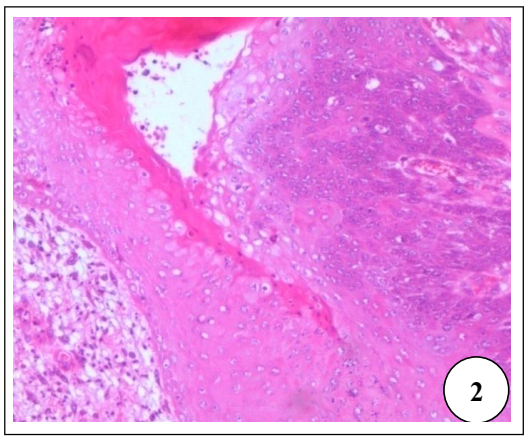

Fig 2: SCC-Moderately differentiated-Multiple Squamous cell layers-H&E-200×.

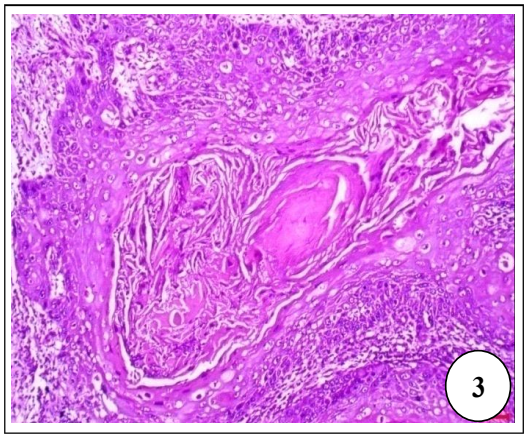

Fig 3: SCC-Well differentiated-Keratin pearls-H&E-200×.

the previous investigations Miyahara *et al.* (2007). The poorly differentiated tumour cases of the present study showed higher MVD when compared to other histological grades. Among poorly differentiated, two cases showed highest mean (PTMVD 32.8±1.64, 33.2±1.92 and ITMVD 16.4±1.14, 17.0±1.58) values. These cases died with in the three month and other three cases showed a mean PTMVD and ITMVD of 32.4±2.07, 31.6±2.60, 31.6±1.67 and 14.0±3.16, 14.6±1.14, 15.2±1.92, respectively. These cases live after six months of surgery and but metastasis occured. Thus, it is found that higher MVD cases died earlier or encountered metastasis shortly when compared to lesser MVD cases. These findings concurred with previous reports of invasive and intratumoural front regions that mentioned an increase in the level of CD 105 with the disease's clinical progress (Martone *et al.* 2005 and Chien *et al.* 2006). The MVD values were different from other authors due to the sample size, difference in the methods of assessing MVD and differences in the selection of hotspots. However, therapeutic significance of or against CD 105 is still unknown and more research on MVD is needed.

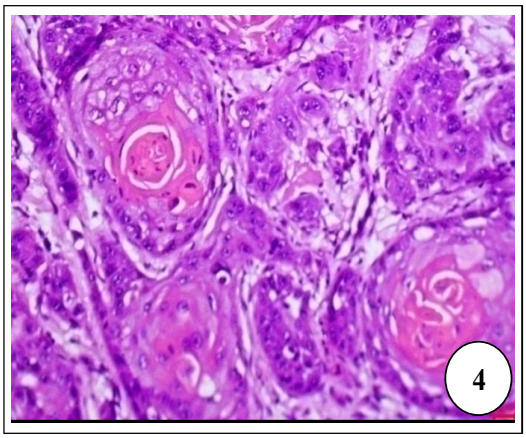

Fig 4: SCC-Well differentiated-Mitotic figures-H&E-400×.

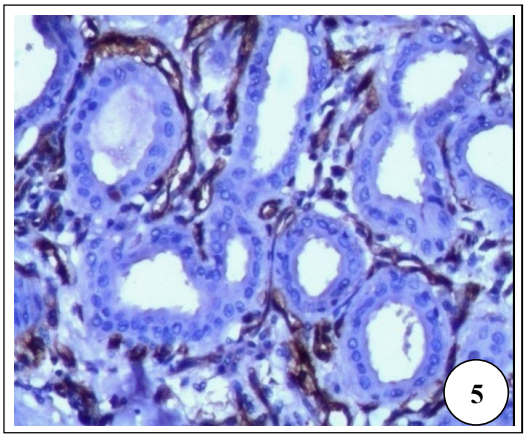

Fig 5: SCC-Moderately differentiated- Hot spot- Brown coloured-IHC-200×.

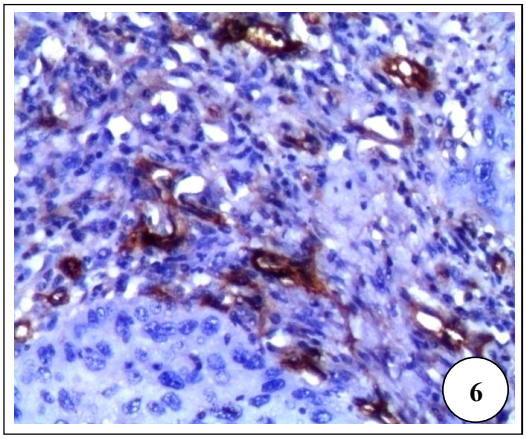

Fig 6: SCC-Hot spot- Interlobular space- Hot spot-Brown coloured- IHC- 200×.

CONCLUSION

The immunohistochemical technique undertaken in the present study was reliable, as the expression of CD 105 was found in all cases and it is consistent with the findings of other studies on SCC. Dogs bearing well differentiated cutaneous tumours of SCC demonstrated a higher MVD and metastasis occured within almost six months (first case 150 days and second case 173 days). Dogs bearing moderately differentiated SCC and having higher MVD died within three months (56 days). Dogs having the highest IMD in poorly differentiated grades died within three months (58 days and 60 days). During diagnosis, MVD had a prognostic value of metastatis propensity, cellular proliferation and histological grade of canine SCC tumours. Therefore, predictive significance was determined in the assessment of angiogenesis of canine cutaneous tumours through MVD. Specifically, the veterinary clinician has better predictive information because of the MVD being determined through CD 105 endothelial marker.

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