EXPRESSION OF MECHANISTIC TARGET OF RAPAMYCIN(mTOR) AND DISHEVELLED, EGL 10 AND PLECKSTRIN DOMAIN CONTAINING MTOR INTERACTING PROTEIN (DEPTOR) IN CANINE SUPERFICIAL AND MAMMARY TUMOURS

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Expression of mechanistic target of Rapamycin (mTOR) and Dishevelled, EGL 10 and Pleckstrin domain containing mTOR interacting protein (DEPTOR) in canine superficial and mammary tumours"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Raimon Mathew

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1. INTRODUCTION

Cell behaviour is modulated by various factors. Lack of nutrients or growth factors hinder the metabolism of cell, with associated altered gene expression required for normal physiology of the cells. The synthesis of protein will also be down regulated, resulting in decreased growth and proliferation. Thus it is evident that, the cells receive the extracellular signals, integrate it, which will trigger multistep intracellular signaling pathways thereby influencing the metabolism and growth of cells. One of the hallmarks of cancer cells is their altered metabolic state where the metabolism will be reprogrammed from a homoeostatic state, where nutrient catabolism or storage prevails, to an anabolic state where nutrients are converted to biomass (Korsheninnikova *et al*., 2006). Hence it is believed that many of the signaling pathways are aberrantly activated in cancer cells. An understanding of these mechanisms that influence cell metabolism and growth, is inevitable in developing a targeted chemotherapy for cancers. One of these pathways is the mechanistic target of Rapamycin (mTOR) signaling pathway, which links nutrients, growth factors and energy availability to cell growth, survival and proliferation.

The story of the mechanistic target of Rapamycin (mTOR) is intimately linked to the discovery of an antifungal agent called Rapamycin, produced by a soil bacteria isolated from the soil samples of Easter Island (Rapa Nui) in 1964. After isolation, the compound was extensively studied and found to have strong anti-neoplastic, immunosuppressive, and anti-proliferative properties (Vezina *et al*., 1975). The exact mechanism of action of Rapamycin remained elusive till 1994, when its mode of action was identified by Brown *et al*. (1994) as an inhibitor of mTOR.

Mechanistic target of Rapamycin (mTOR), an evolutionarily conserved serine/threonine kinase, is a key molecule required for integrating the signals from growth factors, nutrients and stress factors that regulate various cell processes. It was identified that mTOR is essentially required for translation of mRNA, cell-cycle

progression, cell survival and autophagy (Sarbassov *et al*., 2005). It is evident that the mTOR pathway is de-regulated in common diseases including diabetes and cancer, emphasizing the need of understanding the function of the various components of the pathway. Mechanistic target of Rapamycin (mTOR) resides in two different multiprotein complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Guertin and Sabatini, 2007).

Over the last few years, in human oncology, the study of Dishevelled EGL-10 and Pleckstrin domain containing mTOR interacting protein (DEPTOR) has gained importance, due to its role as a natural mTOR regulator (Wang *et al*., 2012). In fact, DEPTOR is an element of both the mTOR complexes and inhibits the kinase activity of mTOR (Peterson *et al*., 2009). Therefore, down regulation of DEPTOR leads to an increased activity of mTOR.

Deregulated mTOR pathway is often associated with the pathogenesis of cancer. Being considered as an endogenous inhibitor of mTOR, the levels of DEPTOR are supposed to be low in tumours. It is consistent with most of the tumours in humans with exceptions like thyroid carcinoma, multiple myeloma (MM) and lung cancer, where DEPTOR was over expressed (Wang *et al*., 2012). Thus it was hypothesized that DEPTOR has a dual role in neoplastic cells, acting either as an oncosuppressor or as an oncogene depending on the tumour type. However, the complete role of DEPTOR in tumourigenesis has not been completely elucidated.

In veterinary medicine, oncotherapy is found to be a challenge, due to the unsatisfactory response to therapy in many animals. Therefore, research on new therapeutic possibilities with better results and less adverse effects are desirable, given the huge increase in number of tumour cases among small animals. Even though, mTOR pathway has been extensively studied in human medicine and even medicines targeting mTOR for cancer therapy are available in the market, studies on this pathway are meager in veterinary medicine. On perusal of the literature, there are

no published reports on the role of DEPTOR and its relationship with the expression mTOR in canine tumours.

Keeping the above facts in view, the present study was designed with the following objectives:-

(a) To assess the expression of mTOR and DEPTOR proteins by immunohistochemistry (IHC) in canine superficial and mammary tumours

(b) To elucidate the relative presence of mTOR and DEPTOR proteins in canine superficial and mammary tumours

2. REVIEW OF LITERATURE

2.1. EPIDEMIOLOGY OF CANINE MAMMARY TUMOURS

Tumours of the mammary gland were the most common tumours in female dogs (Dobson *et al*. 2002, Merlo *et al*. 2008).

Gupta (2008) reported an incidence of 25.85 per cent for canine mammary tumours among all the tumour conditions studied in dogs.

Rezaie *et al*. (2009) concluded that canine mammary tumours (CMTs) were the second most frequent tumours in dogs after skin tumours and were the most common types of neoplasm in female dogs.

Kim *et al*. (2016) observed that one of the most common species that developed mammary neoplasms was canine and could therefore be utilised as an acceptable model for human breast tumour.

Do-Carmo *et al*. (2019) reported that mammary tumours represented about 50 to 70 per cent of all tumours in female dogs and their incidence directly correlated with the age and reproductive status of the dog.

2.1.1. Age-wise occurrence

Bostock (1986) reported that there was a remarkable increase in occurrence of mammary neoplasms in canines with advancing age, with the highest occurrence at 11 years, followed by a mild decrease thereafter.

Sandhu (1995) reported that the dogs aged between six to ten years had the peak incidence of mammary tumours and the dogs less than four years had shown lowest incidence.

Gill (1997) reported mammary tumours in dogs aged between two to fourteen years. The highest frequency (27.27 per cent) observed in the age group of eight to twelve years followed by six to eight years (24.24 percent), 10-12 years (12.21 per cent) and the lowest frequency in the age group of 12-14 years, two to four years and less than two years (3.03 per cent each).

Nayyar (2002) studied mammary tumours in canines in the age group of two to 15 years and reported that the average age of the dogs affected with mammary tumours was 9.6 years and that the dogs aged between 8-10 years had shown the highest incidence (22.22 per cent) followed by 12-14 years (19.44 per cent), whereas, dogs aged between two to four years had shown lowest incidence (2.78 per cent) and no case occurred in dogs less than two years of age.

Chang *et al*. (2005) reported that middle aged dogs were more prone to mammary tumours and the median age of occurrence was between eight and ten years.

Sorenmo *et al*. (2009) observed that malignant mammary tumours were more frequent in older dogs. They found that the mean age of the dogs with malignant and benign tumours was 9.5 years and 8.5 years respectively. They had also reported that the benign mammary tumours were smaller in size when compared with the malignant counterparts.

Do-Carmo *et al*. (2019) concluded that there was a significant positive correlation between the age of the dogs and occurrence of malignant mammary tumours.

2.1.2. Gender-wise occurrence

Moulton (1970) observed that mammary tumours were specific tumours of females, often attributed to hormonal abnormalities, and were rare in males.

Dhami *et al*. (2010) in their study found that, out of the 63 cases of mammary tumour in canines, 60 were observed in females and three in males.

Gupta *et al*. (2012) observed that out of the 51 cases of mammary tumours, only one case was seen in a male dog.

2.1.3. Breed-wise occurrence

Owen (1991) observed the highest incidence of mammary tumours in Dachshunds and Cocker spaniels, while Chihuahuas had the lowest risk.

Sandhu (1995) and Gill (1997) reported the highest incidence of mammary tumours in Spitz followed by Doberman Pincher.

Goldschimdt (1998) concluded that small breeds like Chihuahua, Poodle, Maltese and Yorkshire terrier had high incidence of benign mammary tumours. For malignant mammary tumours, Afghan hound, English setter and Miniature poodle had highest predilection. Golden retriever, Boxer and Rottweiler had the least risk of developing mammary tumours.

Bala (2005) reported highest predisposition of CMTs in Samoyed/white spitz (38.46 per cent) followed by Labrador (23.07 per cent), German shepherd (19.23 per cent), Doberman (7.69 per cent), Non-descript (7.69 per cent) and Dalmatian (3085 per cent).

Shivani (2007) observed the highest incidence of CMTs in Pomeranian followed by German shepherd and Labrador Retriever.

Reddy *et al*. (2009) reported the highest incidence of CMTs in German shepherd (25.0 per cent) followed by Spitz (24.22 per cent), Non-descript (19.53 per cent), Pomeranian (10.94 per cent), Labrador (6.25 per cent), Boxer (3.91 per cent), Doberman (4.69 per cent), Cocker Spaniel (3.13 per cent), Bhutia (1.56 per cent) and Great Dane (0.78 per cent).

Salas *et al*. (2015) reported that poodles have the highest incidence of mammary tumours in the small breed category where as German shepherd had the highest incidence among the large breeds.

Do-Carmo *et al*. (2019) observed that breed predisposition to mammary tumours was not well established in the literature, which could be due to the difference in profile of the dog population in various studies.

2.1.4. Tumour-wise occurrence

Hampe and Misdorp (1974) reported that almost half of the CMTs were malignant.

Lana *et al*. (2007) concluded that most of the malignant canine mammary tumours were carcinomas and less than five per cent were sarcomas.

Stratmann *et al*. (2008) observed that the most common benign mammary tumour was adenoma and the most common malignant tumour was carcinoma.

Filho *et al*. (2010) observed that the most frequent histologic type of mammary tumour was simple carcinoma, both in necropsy and biopsy samples.

Horta *et al*. (2014) in their study observed that benign mixed tumour was the most frequent (56.2 per cent) benign mammary tumour and the most frequent malignant neoplasm was carcinoma in mixed tumour (47.5 per cent).

2.2. GROSS PATHOLOGY OF CANINE MAMMARY TUMOURS

Kumar *et al*. (2011) observed that canine mammary tumours were usually elliptical to round in shape with consistency varied from soft to hard and cut surface showed grayish white colour. Some neoplasms were cystic, filled with sero sanguineous fluid and some had cartilaginous structures.

Yogitha *et al*. (2015) reported that the gross morphological features of the tumours helped them to identify mixed mammary tumours, as they appeared lobular in shape and consisted of bone or cartilage which imparted hard and rigid consistency to the tumour.

Gabli *et al*. (2017) evaluated the size of the CMTs in 215 cases and reported that the mean size was 5.4±0.4 cm.

2.3. HISTOPATHOLOGY OF CANINE MAMMARY TUMOURS

Misdorp *et al*. (1972) concluded that simple carcinomas consisted of only one type of cells, either myoepithelial cells or luminal epithelial cells. They had observed that tubular carcinoma consisted of neoplastic cells arranged in tubular pattern, whereas in tubulopapillary carcinoma the tubules were arranged in a pedunculated and papillary fashion.

Misdorp *et al*. (1973) observed that in solid carcinoma, the neoplastic cells were arranged in solid sheets without lumina.

Misdorp (2002) reported that the histopathological classification of canine mammary tumours by the World Health Organisation (WHO) was the only reference document for classification of CMTs by pathologists for decades. This classification consisted of six benign and 14 malignant categories.

Karayannopoulou *et al*. (2005) endorsed the practical utility of the histological classification system of CMTs by WHO and opined that the classification could be effectively used as a prognostic indicator.

De-Las *et al*. (2005) reported that there was no significant relation between the histological subtype and survivability variables in CMTs.

Gama *et al*. (2008) reported that micropapillary mammary carcinomas were characterised by papillary cell clusters surrounded by lacunar spaces which were empty and the papillae were deficient of true fibrovascular core.

Goldschmidt *et al*. (2011) presented an alternative scheme for histological classification of CMTs. This classification system was based on the architectural details of the neoplastic cells and the involvement myoepithelial cells in the tumour process. Accordingly, the CMTs were categorized into 23 malignant and seven benign variants.

Goldschmidt *et al*. (2011) observed that in carcinoma arising in benign mixed tumour (CABMT), the benign part was detectable in the section but, multifocally there were areas of epithelial cells with marked pleomorphism and increased mitoses (foci of carcinoma cells).

Im *et al*. (2014) opined that the 2011 classification system of CMTs by Goldschimdt *et al*. (2011) was exhaustive and complete.

Alonso-Diezz *et al*. (2019) described that in spindle cell variant of carcinoma the neoplastic cells and nuclei were in fusiform shape. The nucleoli were prominent and the cytoplasm was eosinophilic There was moderate anisocytosis and anisokaryosis.

Mathew *et al*. (2019) observed that fibroadenoma had tubules which were lined by cuboidal cells with uniform sized round nuclei and the tubules were surrounded by loose connective tissue. They also observed that the comedocarcinoma was a variant of solid carcinoma, with an area of necrosis in the centre of neoplastic cell clusters.

2.4. GRADING OF CANINE MAMMARY TUMOURS

Bloom and Richardson (1957) described a comprehensive numerical grading method for human breast cancers. The grading method was successful in assessing the malignancy of the breast tumours from the histological characteristics. The extend of tubule formation, hyperchromasia and pleomorphism of the nuclei and the number of mitoses per 10 high power field were the three factors used for the grading. A

score of one to three was assigned to each factor and finally a combined score was obtained by adding the individual values. Accordingly, they classified the human breast cancers into Grade I (Low grade), Grade II (Intermediate grade) and Grade III (High grade) when the final combined scores were three to five, seven to eight and eight to nine respectively.

Elston and Ellis (1991) described a refined version of the Bloom and Richardson method, popularly called as the Nottingham histological grade (NHG). Unlike the previous method, besides the nuclear pleomorphism, the Nottingham method expressed the tubule formation in percentage and only the number of mitoses was evaluated but the hyperchromatic nuclei were excluded.

Grading of CMTs was a challenge as far as any veterinary oncologist is concerned, owing to the heterogeneity of histological presentation and the diversity in cell population, which included luminal epithelium, mesenchymal part and myoepithelium. To overcome this, Clemente *et al*. (2010) proposed the canine adaptation of human Nottingham histological grading system (ca-NHG) which included modification in assessment of mitotic count and nuclear pleomorphism. They evaluated the tubule formation in the most representative malignant area in case of heterogeneous CMTs, where as in case of complex or mixed tumours, tubular scoring was carried out only on the epithelial areas, but the nuclear pleomorphism was evaluated in all the malignant components.

Recently different studies have confirmed that the canine adapted Nottingham histological grading provided independent prognostic indication (Pena *et*. *al*., 2012, Mainenti *et al*., 2014, Carvalho *et al*., 2016)

2.5. EPIDEMIOLOGY OF CANINE SUPERFICIAL TUMOURS

Tumours affecting the skin and subcutaneous tissues were very common in dogs. It accounted for nearly 33 per cent of all tumours seen in dogs (Finne and Bostoc 1979, Bostoc, 1986).

Bastianello (1983) reported that skin was the organ which was most commonly affected by tumours in dogs.

2.5.1. Age-wise occurrence

Kaldrymidou *et al*. (2002) observed that the age group of the dogs reported with skin tumours ranged between four months to 16 years and the average age of occurrence was approximately nine years.

Pakhrin *et al*. (2007) concluded that the mean age of the dogs affected with skin neoplasms was 8.3 years.

Dayananda *et al*. (2009) reported the occurrence of skin tumours in dogs in the age group of five to 18 years with the mean age of occurrence as 7.72 years.

Kashyap *et al*. (2013) reported that the mean age of the dogs affected with malignant and benign cutaneous neoplasms were 10 years and 6.79 years respectively.

2.5.2. Gender-wise occurrence

Sanja *et al*. (2005) in their study observed 58.3 per cent of cutaneous neoplasms in male dogs and 41.7 per cent in female dogs.

Dayananda *et al*. (2009) observed higher incidence of skin tumours in males (72.95 per cent) than in females (29.05 per cent).

Naick (2013) observed that among the dogs reported with skin tumours 57.14 per cent were males while 42.86 per cent were females.

Simenov (2014) reported that the sex predilection of round cell tumours of skin and soft tissues in canines was not significantly different (male 46.08 per cent and female 53.92 per cent).

2.5.3. Breed-wise occurrence

Dayananda *et al*. (2009) reported the highest incidence of superficial tumours in Non-descript breed followed by German shepherd and Labrador.

Babu *et al*. (2012) observed that the breed commonly affected with superficial tumours was German shepherd followed by Mongrels, Labrador and Rottweiler.

Lima *et al*. (2013) reported that Rottweiler was the most susceptible breed for squamous cell carcinoma and digit was the commonly affected part of the body.

Naick (2013) observed highest incidence of superficial tumours in German shepherds (32.14 per cent) followed by non-descript breed (17.85 per cent). Labrador, Rottweiler and Doberman had an incidence of 14.25, 10.71 and 7.14 per cent respectively.

2.5.4. Tumour-wise occurrence

Chalita *et al*. (2001) observed that 38 per cent of the skin tumours in dogs were epithelial and melanocytic in origin whereas 30 per cent were of mesenchymal origin.

Kaldrymidou *et al*. (2002) reported that around 40 per cent of the skin and subcutaneous tumours in dogs were malignant.

Guzman *et al*. (2003) reported that adverse environmental factors, like exposure to ultraviolet rays in sunlight were the major cause of skin cancer development.

Mukaratirwa *et al*. (2005) observed that increased number of malignant skin tumours occurred in the trunk and limbs in dogs than other sites.

Sanja *et al*. (2005) reported that out of the 211 skin tumours in dogs, 58.2 per cent were epithelial and melanocytic tumours, 22.7 per cent were haematopoetic tumours and 19.43 per cent were soft tissue tumours.

Belluco *et al*. (2013) observed that 47.4 per cent of all the malignant canine digital lesions were squamous cell carcinomas and the frequency of its incidence in forelimb was twice when compared with the hind limb.

Kashyap *et al.* (2013) observed that 40 per cent of the reported skin tumours in dogs were malignant and the rest 60 per cent were benign.

Simkus *et al*. (2016) observed that among the total skin tumours, epithelial and melanocytic tumours accounted for 52.3 per cent and mesenchymal tumours constituted 47.7 per cent.

2.6. GROSS PATHOLOGY OF CANINE SUPERFICIAL TUMOURS

Ozyigit *et al*. (2005) observed that, on gross examination sebaceous adenoma was a whitish mass with nodular appearance and firm consistency.

Chandrashekaraiah *et al*. (2011) after reviewing 17 cases of squamous cell carcinoma in dogs reported that grossly, they varied in size, appeared as ovoid, round, irregular or cauliflower like masses with superficial ulcerations. The tumours had rough surface and the colour varied from pink to light brown.

Headley *et al*. (2011) reported myxosarcoma on the right scapular region in a dog which was a large circumscribed subcutaneous mass with soft consistency, which invaded the adjacent muscle.

Sawale *et al*. (2014) reported a haemangioma at the base of tail in a dog as a single growth of 2x2 cm size. The mass was red in colour with firm consistency and contained blood.

Venugopal *et al*. (2014) observed that grossly, hepatoid adenoma was firm, circumscribed and haemorrhagic and the dog evinced pain on palpation.

Rai and Chadrapuria (2015) recorded that lipoma was a soft and round tumour on the lateral aspect of left thigh which was painless on palpation.

Raval *et al*. (2015) reported that the hair follicle tumours were well demarcated from the surrounding tissues and multilobulated.

Hendrick (2017) observed that fibromas occurred most commonly on the head and limbs of dogs. They were round or oval with firm, rubbery consistency and grayish white colour on cut surface.

Nishiya *et al*. (2016) observed that cutaneous melanocytomas were usually small, pigmented, solitary, firm, and freely moveable over deeper structures.

2.7. HISTOPATHOLOGY OF CANINE SUPERFICIAL TUMOURS

On histological examination, sebaceous adenoma had circumscribed lobules of varying size. These lobules consisted of 2 cell types, peripherally placed undifferentiated basaloid cells and centrally placed differentiated sebaceous cells. Anisocytosis, mitotic figures and inflammatory cells were absent (Ozyigit *et al*. 2005).

Jasik *et al*. (2009) observed that, the trichoblastomas consisted of basaloid cells with hyperchromatic nuclei and granular eosinophilic cytoplasm depending on the subtype of the tumour. In ribbon type trichoblastoma, the neoplastic cells formed long cords of cells that branched and joined together. The cords of cells were radiating from a central island of densely packed cells in medusoid subtype. In cystic variant of trichoepithelioma several cystic areas lined by multilayered epithelium were seen.

Ribeiro *et al*. (2009) observed that fibrosarcoma consisted of interwoven bundles of fibroblast-like cells with oval nuclei which were hyperchromatic. Histological variations of the tumour were observed with areas of closely packed spindle cells alternating with areas which were less populated with cells and had dense fibers.

In myxosarcoma, the cells were spindle shaped and plump, haphazardly distributed and were bathed in a rich mucopolysaccharide matrix. The neoplastic epithelial cells had indistinct margins, pale eosinophilic cytoplasm and marked cellular and nuclear pleomorphism (Headley *et al*., 2011).

Belluco *et al*. (2013) observed that in the well differentiated squamous cell carcinomas, cells were arranged in a lobular pattern with squamous differentiation and presence of keratin pearls. In the moderately differentiated type, lobules were smaller and keratin was scant. The undifferentiated squamous cell carcinomas had an anaplastic morphology with the polygonal cells arranged in cords. Keratin formation was absent in undifferentiated types.

Balachandran *et al*. (2014) reported that histological examination of haemangiomas revealed variable sized and dilated spaces containing numerous blood cells. The neoplastic cells were spindle shaped with eosinophilic cytoplasm and vesicular nuclei.

Huppes *et al*. (2016) observed that histologically lipoma had resemblance with normal adipose tissue and the adipocytes were of the same size or slightly bigger than the normal ones.

Yumusak *et al*. (2016) observed that in hepatoid gland adenoma, the epithelial cells were hepatocyte like with eosinophilic cytoplasm and large vesicular nuclei with prominent nucleolus organised in the form of islets.

Henrick (2017) observed that fibroma was characterised by collagenous fibers which were arranged in interwoven fascicles. The neoplastic fibrocytes were low in number compared with the abundant collagen.

2.8. MECHANISTIC TARGET OF RAPAMYCIN (mTOR)

Vezina *et al*. (1975) extracted a compound from a streptomycete obtained from the soil sample of South Pacific island of Rapa Nui (also known as Easter

island) with remarkable immunosuppressive, anti-fungal and anti-tumour properties. The compound was named as Rapamycin.

Chung *et al*. (1992) demonstrated that Rapamycin blocked the activation of 70 kilo Dalton S6 protein kinases, by forming a complex with the peptidyl-prolylisomerase FK-506 binding protein (FKBP12) to inhibit signal transduction pathways required for cell proliferation and growth.

Sabers *et al*. (1995) identified that the main target of Rapamycin-FKBP12 complex was mechanistic target of Rapamycin (mTOR).

Cardenas *et al*. (1999) described in budding yeast, two targets of Rapamycin (TOR), namely TOR1 and TOR2, which controlled many growth related processes in response to nutrients and favourable environmental conditions.

Hara *et al*. (2002) reported that mechanistic target of Rapamycin complex 1 (mTORC1) was composed of three core components: mTOR, Raptor (Regulatory-associated protein of mTOR) and mLST8 (Mammalian lethal with Sec13 protein 8 also called as GßL).

Jacinto *et al*. (2004) reported that mechanistic target of Rapamycin complex 2 (mTORC2) also had mTOR and mLST8, but instead of Raptor, it had Rictor (Rapamycin insensitive companion of mTOR).

Murakami *et al*. (2004) described that mTOR is integral to all cells and play a crucial role in cell proliferation and growth.

Sarbassov *et al*. (2005) showed that mTORC2 had an important role in the phosphorylation and activation of Protein kinase B (Akt), which is the main effector of insulin/PI3K signaling. The activated Akt promoted cell growth, survival and proliferation of cells.

Mechanistic target of Rapamycin complex 1 (mTORC1) enhanced protein synthesis, which was required for the growth and division of cells, by phosphorylating two key proteins, p70S6 kinase1 (S6K1) and eukaryotic initiation factor 4E binding protein (4EBP). (Dorello *et al*., 2006).

Garcia-Martinez and Alessi (2008) reported that mTORC2 controlled the phosphorylation and activation of S6K1 which regulated the transport of ions and survival of cells.

Porstmann *et al*. (2008) described that mTORC1 enhanced the *de-novo* lipid synthesis through sterol responsive element binding protein (SREBP), resulting in fatty acids and cholesterol biosynthesis, which was essential for new membrane formation and expansion of cells.

Duvel *et al*. (2010) observed that mTORC1 enhanced the translation of the transcription factor, hypoxia inducible factor 1 (H1F1α) resulting in increased expression of phospho-fructokinase (PFK) that promoted gycolysis resulting in nutrient incorporation into new biomass.

In addition to activation of various anabolic processes, mTORC1 also promoted the growth of cell by suppression of catabolism of proteins, mostly autophagy. Nutrient replete conditions drove mTORC1 to phosphorylate unc-51-like kinase 1 (ULK1), resulting in prevention of autophagosome formation. (Kim *et al*., 2011)

Gao *et al*. (2012) reviewed that multiple signals such as nutrients, growth factors, energy status, and various stress factors activated the mTORC1 which resulted in promotion of protein synthesis, cell survival, cell proliferation, ribosome biogenesis, angiogenesis, cell migration, invasion and metastasis by phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1). mTOR complex 2 functions in actin remodeling, cell-cycle progression, and cell survival through the regulation of protein kinase C*α* (PKC*α*) and glucocorticoid-induced protein kinase 1 (SGK1) (Fig. 1.)

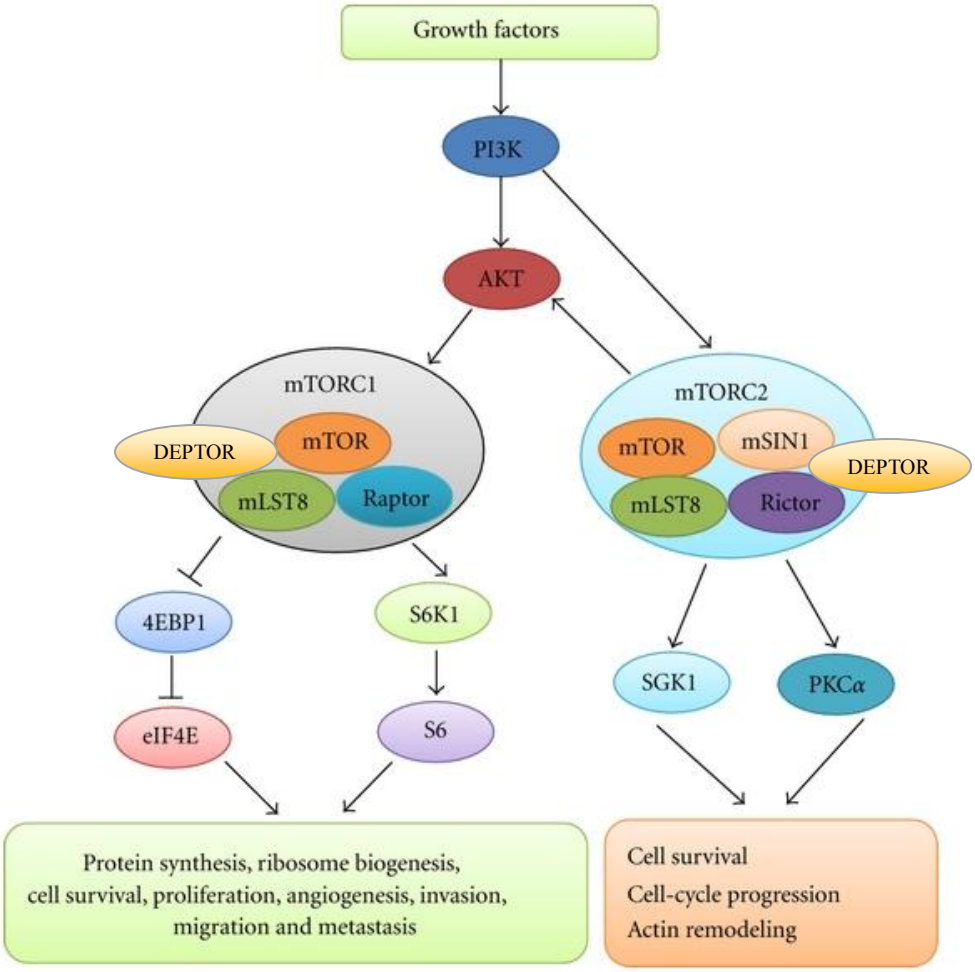

Fig. 1. mTOR signaling pathway. PI3K: phosphatidylinositol-3-kinase; AKT: protein kinase B. mTOR: mechanistic target of rapamycin; mTORC1: mTOR complex 1; mTORC2: mTOR complex 2; Raptor: regulatory-associated protein of mTOR; Rictor: rapamycin-insensitive companion of mTOR; mSIN1: mammalian stress-activated protein kinase interacting protein; eIF4E: eukaryotic initiation factor 4E; 4EBP1: eIF4E -binding protein 1; S6K1: S6 kinase 1; SGK1: glucocorticoid-induced protein kinase 1; PKCα: protein kinase Cα.

Ben-Sahra *et al*. (2016) concluded that mTORC1 promoted the production of nucleotides required for DNA replication and ribosome biogenesis in dividing cells by enhancing the Activating transcription factor-4 (ATF4) dependent expression of Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), which was the key agent of mitochondrial tetrahydrofolate cycle that gave one carbon units for purine synthesis.

2.8.1. Role of mTOR in cancer

Bachman *et al*. (2004) showed that the phosphatidylinositol 3-kinase catalytic subunit (*PIK3CA*) which is a subunit of PI3K was the most mutated oncogene in human breast cancer resulting in epithelial carcinogenesis.

Saal *et al*. (2005) demonstrated that almost 35 per cent of the human breast cancers had loss of Phosphatase and tensin homolog (PTEN) expression, which is an inhibitor of PI3K/Akt/mTOR pathway. Thus they opined that this pathway could be potentially targeted for anticancer therapy.

The signaling of mTORC2 had a major role in cancer, since it could activate Akt, which drove various pro-proliferative processes such as glucose intake and glycolysis besides inhibiting apoptosis (Guertin *et al*. 2009).

Sato *et al*. (2010) described the role of mTOR in tumourigenesis, as mTOR mutations have been observed in variety of human cancers.

Hsieh *et al*. (2012) reported that mTORC1 driven phosphorylation of 4EBP1 was critical in tumourigenesis in mouse models of T cell lymphoma and prostate cancer.

2.8.2. Role of mTOR in canine tumours

Gordon *et al*. (2008) evaluated the expression of mTOR and its downstream effector p70S6 kinase in canine osteosarcoma cell lines and concluded that both the proteins were expressed in canine osteosarcoma cells.

Kent *et al*. (2009) evaluated the expression of total and phosphorylated Akt, mTOR and p70S6 kinase 1 (p70S6K), by western blot analysis in primary canine malignant melanoma cell lines and concluded that all the three proteins were expressed in canine melanoma cells. They had also demonstrated that when the melanoma cell lines were exposed to Rapamycin, which is an mTOR inhibitor, the mTOR pathway activation was inhibited.

Murai *et al*. (2012) demonstrated by immunohistochemistry that the mTORC2/Akt/4E-BP1 pathway was activated in canine haemangiosarcomas in contrast to that of haemangiomas where as the mTORC1 was activated in endothelial cells but not in haemangiosarcomas and concluded that mTORC2/Akt/4E-BP1 pathway was regulated independently of mTORC1 and could be used as a potential target for therapy in canine haemangisarcomas.

Rodriguez *et al*., (2012) conducted a study on 25 canine mast cell tumours and demonstrated that all the tumours had active expression of Akt which is an upstream activator of mTOR. Thus they have concluded that the Akt-mTOR pathway played a crucial role in the development of canine mast cell tumours.

Delgado *et al*. (2015) studied the phospho-mTOR expression in CMTs by immunohistochemistry and concluded that it was not expressed in normal mammary gland but cytoplasmic expression of the protein was observed in 78 per cent of the canine mammary carcinomas which was suggestive of the critical role of phospho-mTOR activation on neoplastic transformation of mammary gland.

2.9. DISHEVELLED, EGL10 AND PLECKSTRIN DOMAIN CONTAINING MTOR INTERACTING PROTEIN (DEPTOR)

Chen and Hamm (2009) reported that DEPTOR is a 49 kDa protein coded by the *DEPTOR* gene and that it has got three domains, two Dishevelled, Egl-10, Pleckstrin (DEP) which were required for membrane association of signaling

proteins, and a Postsynaptic density 95, Discs large, Zonula occludens-1 (PDZ) which was important for protein-protein interaction.

Peterson *et al*. (2009) stated that DEPTOR, which is a component of both mTORC1 and mTORC2, regulated the mTOR activity negatively by inhibiting its kinase activity. They also observed that a negative feedback loop exists between DEPTOR and mTOR. Thus, down-regulation of DEPTOR led to increase in activity of mTOR.

The PI3K signaling was found to be overactive in tumours, which represses DEPTOR levels. Hence *DEPTOR* mRNA expression was found to be low in most of the tumours. However an over expression of DEPTOR was observed in multiple myeloma (MM) cells, which resulted in reduced endoplasmic reticulum stress and subsequent survival of MM cells. Thus it was concluded that DEPTOR acted as an oncogene in MM cells (Peterson *et al*., 2009).

Pei *et al*. (2011) demonstrated that DEPTOR was over expressed in human patients with differentiated thyroid carcinoma and the expression correlated with early recurrence and poor prognosis.

Li *et al*. (2014) reported that DEPTOR expression was not at all observed in pancreatic ductal adenocarcinoma (PDAC) and opined that DEPTOR had tumour suppressor effect in PDAC.

Obara *et al*. (2015) concluded that metformin; an anticancer drug for liver cancer exerted its effect through activation of DEPTOR and subsequent mTOR suppression in human liver cancer cells.

Parvani *et al*. (2015) reported that DEPTOR expression was absent in low-grade human MCF10aCa1h and high-grade human MCF10aCa1a mammary carcinoma cells relative to their indolent human MCF10aT1K counterparts. Similarly, DEPTOR expression was also dramatically reduced in metastatic MDA-MB-231 cells as compared to non-metastatic MCF7 cells. Collectively, they opined that

diminished DEPTOR expression was characteristic of invasive human mammary carcinomas.

Zhou *et al*. (2016) concluded that DEPTOR acted as a tumour suppressor in human lung adenocarcinoma by inhibiting the EGFR (epidermal growth factor receptor) mediated mTOR phosphorylation and progression of the tumour.

Ryan *et al*. (2019) reported a five-fold reduction in DEPTOR expression in cultured glioblastoma cells resulting in mTOR over expression and enhanced anabolism of the tumour cells.

2.10. IMMUNOHISTOCHEMISTRY

Coons *et al*. (1941) demonstrated that antigens especially microorganisms could be localized in tissues and could be visualized by using antibodies. They used β-anthryl derivative of antipneumococcus III rabbit antibody to localize and visualize the Type III pneumococci by fluorescence microscopy.

The publication of the research paper by Coons *et al*. in 1941 describing the technique of detecting antigens in cells by immunofluorescence marked the beginning of Immunohistochemistry (IHC). It became a valuable tool in both research and diagnosis of infectious diseases and tumours in animals. Immunohistochemistry is an amalgamation of three scientific disciplines namely, immunology, histology and chemistry (Ramos-Vara, 2005).

Immunohistochemistry was reported as one of the most important ancillary techniques for the characterization of neoplasms in veterinary medicine (Ramos-vara *et al*., 2008).

Immunohistochemistry is a technique in which the tissue or cellular components (antigens) are identified by means of antigen-antibody interactions. The antibody binding site can be identified by direct labeling of the antibody or by use of a secondary antibody (Sanderson *et al*., 2019)

2.10.1. Immunohistochemistry for demonstration of mTOR and DEPTOR

Li *et al*. (2012) demonstrated the expression of mTOR on formalin fixed paraffin embedded (FFPE) tissues of gastric carcinoma in human patients using rabbit monoclonal anti-mTOR antibody.

Murai *et al*. (2012) demonstrated the mTOR expression in a series of haemangiomas and haemangiosarcoma in dogs.

Delgado *et al*. (2015) studied the expression of phospho-mTOR, the activated form of mTOR, in canine mammary tumours by IHC using anti-phospho-mTOR rabbit monoclonal antibody. They observed that 78 per cent of the cases showed expression of the protein.

Liu *et al*. (2015) demonstrated the high expression of DEPTOR in human oesophageal squamous cell carcinoma by IHC.

3. MATERIALS AND METHODS

The study on "Expression of mechanistic target of Rapamycin (mTOR) and Dishevelled, EGL10 and Pleckstrin domain containing mTOR interacting protein (DEPTOR) in canine superficial and mammary tumours" was conducted at the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

3.1. SAMPLES

Excision biopsy samples of mammary and skin tumour suspected growths from dogs presented to University Veterinary hospitals, Mannuthy and Kokkalai were collected for the study during the period from January 2018 to March 2019. Out of the 129 different tumour cases reported, 22 were mammary tumour growths and 17 were superficial tumour growths.

The tumour samples were fixed in 10 per cent neutral buffered formalin for histopathology and immunohistochemistry. Clinical history of the animals affected with the tumour, description of the lesions pertaining to size, shape, colour and location were recorded at the time of tissue collection.

3.2. EPIDEMIOLOGY OF THE TUMOURS

The age-wise, breed-wise, sex-wise and tumour-wise occurrence of canine mammary and superficial tumours was studied based on the clinical history of the animals.

3.3. GROSS PATHOLOGY

Grossly, the tumours were examined for the size using a vernier caliper, shape and colour by visual examination and consistency by touch.

3.4 HISTOPATHOLOGY

Tumour tissues fixed in 10 per cent neutral buffered formalin were processed by routine paraffin embedding method (Spencer and Bancroft, 2013). Paraffin embedded blocks were sectioned at four µm thickness using a rotary microtome and were stained by routine Haematoxylin and Eosin staining procedure (Bancroft and Layton, 2013).

3.4.1. Classification of canine mammary tumours

The benign and malignant mammary tumours were classified into different subtypes based on the histological features as described by Goldschmidt *et al*. (2011).

3.4.2. Grading of canine mammary tumours

The histological malignancy grading (HMG) was done using the modified numeric method of Elston and Ellis for grading human breast cancers, adapted to grade canine mammary tumours (Clemente *et al*., 2010). The details are described below :-

3.4.2.1. Tubule formation

Tubule formation in the section was assessed semi quantitatively and a score of one point was given when more than 75 per cent of the area was composed of definite tubules. Two and three points were given respectively when 10-75 per cent and < 10 per cent of the area was covered by tubules.

3.4.2.2. Nuclear pleomorphism

When the nuclei were small with minimum variation in size and had uniform chromatin, a score of one point was given. Nuclei larger in size with moderate anisokaryosis were given 2 points. When nuclei were vesicular and varying considerably in size and shape and bearing prominent nucleoli, 3 points were given.

3.4.2.3. Mitotic counts

Mitotic activity was assessed at a magnification of 400x (high power field, HPF), which provide a field area of 0.237 sq.mm. A minimum of 10 fields were examined. Upto nine mitoses per 10 HPF were given one point, 10-19 mitotic figures per 10 HPF were scored two points and 20 or more mitotic figures per 10 HPF were given three points.

After scoring in each of the above aspects, the scores were added to get a number between three and nine. Then the grade was allocated as below:-

HMG I (low grade) - three to five points, well differentiated

HMG II (intermediate grade) - six or seven points, moderately differentiated

HMG III (high grade) - eight or nine points, poorly differentiated

3.5. IMMUNOHISTOCHEMISTRY

Expression pattern of the proteins, mTOR and DEPTOR in the formalin fixed paraffin embedded (FFPE) tissues was studied by immunohistochemistry as per technique elaborated by Ramos-Vara, (2005). The antibodies used to detect the proteins were mTOR rabbit monoclonal antibody (Ser. No. 2983, clone 7C10) and DEPTOR rabbit monoclonal antibody (Ser No. 11816, clone D9F5) which were procured from Cell Signaling Technology, Massachusetts, United States.

3.5.1. General Material

3.5.1.1. Glassware and Plastic ware

Properly cleaned neutral glassware (Borosil) and plastic ware were used for immunohistochemistry procedure.

3.5.1.2. Preparation of adhesive coated slides

One end frosted slides procured from Himedia, Mumbai were rinsed in acetone for 2 minutes and then air dried. The slides were immersed in 2 per cent 3-Aminopropyl triethoxysilane in acetone for 10 – 15 minutes, followed by wash in two

changes of distilled water. The slides thus prepared were dried at room temperature and stored in slide boxes for future use.

3.5.2. Immunohistochemistry protocol

3.5.2.1. Deparaffinisation and dehydration

Sections of four µm thickness of tumour tissues were collected on to the adhesive coated slides. The sections were heat fixed by placing in an oven at 37°C overnight. After warming the slides at 45°C for 30-45 minutes, deparaffinisation of the sections was carried out by immersing in two changes of xylene for ten minutes each. The deparaffinised sections were hydrated to deionized water after immersing in 100 per cent, 80 per cent, 70 per cent and 50 per cent isopropyl alcohol; each for five minutes duration.

3.5.2.2. Antigen retrieval

The slides were taken out from deionized water and blotted well using a blotting paper. They were then immersed in a couplin jar containing citrate buffer (pH 6.0). The jar was closed with a loose lid and was centered in a boiling water bath. The slides were heated in the buffer for 20 minutes whose temperature was raised to 95°C. The sections were then allowed to cool in the buffer for another 20 minutes at room temperature. The sections were then washed thrice with deionised water and 1 per cent Tris buffered saline-Tween 20 buffer (1 per cent TBST).

Composition of citrate buffer (pH 6.0)

Trisodium citrate dihydrate - 2.9 g
Double distilled water – 1000 ml
Adjust pH to 6.0 with citric acid
Add 0.5 ml of Tween 20

Composition of Tris buffered saline stock solution (10 per cent TBS)

Tris base – 24g

Sodium chloride – 80g

Double distilled water – 900ml

Adjust pH to 7.6 with concentrated Hydrochloric acid

Make upto one litre with distilled water

Composition of Tris buffered saline – Tween 20 working solution (one per cent TBST)

10 per cent TBS – 100ml

Double distilled water – 900ml

Tween 20 – 0.5ml

3.5.2.3. Antibody dilution

Anti-mTOR and anti-Deptor antibodies were diluted to a concentration of 1:100 and 1:500 respectively with primary antibody dilution buffer.

Composition of primary antibody dilution buffer – one per cent BSA (For 10 ml volume)

10 per cent TBS – 1ml

Distilled water – 9ml

Bovine serum albumin – 0.1g

Tween 20 - 10µl

3.5.2.4. Immunohistochemical staining

After antigen retrieval and washing with one per cent TBST, the PAP pen (abcam, Massachusetts, United States) was used to create a hydrophobic barrier around the tissue sections to localize the staining reagents to the tissue sections only. The Super Sensitive™ RTU Polymer-HRP IHC Detection Kit (QD400-60KE, Biogenex, California, United States) was used for immunohistochemical staining. Hundred microlitre of peroxide block (3 per cent hydrogen peroxide) supplied with the kit was then added to the sections and incubated at room temperature for 20 min.

After incubation, the slides were rinsed well with one per cent TBST. The tissue sections were then treated with Power Block™ and incubated at room temperature for 20 min for blocking the non-specific protein-protein interactions. After incubation, the slides were not washed, but blotted with a blotting paper. The sections were then incubated with 30 – 50 µL of primary antibody (anti-mTOR and anti-Deptor) at 4°C for 12 hours. The slides were washed thoroughly in one per cent TBST after incubation. The sections were then incubated with 30 µl of Super Enhancer™ reagent at room temperature for 20 minutes to enhance the signal, followed by washing with one per cent TBST. Next step was to incubate the sections at room temperature for 30 min with 100 µL Polymer-HRP Reagent, provided with the kit by the name SS label. After incubation, the slides were washed in one per cent TBST. The entire antibody-enzyme complex was then made visible by incubation with a chromogenic substrate Diaminobenzidine (DAB), (Cell Signaling Technology, Massachusetts, United States). Diaminobenzidine substrate solution was prepared by adding 30 µL DAB chromogen to one ml of DAB substrate followed by thorough mixing. The prepared DAB substrate solution was applied on to the sections and incubated for one to ten min (depending on colour development) at room temperature followed by washing with buffer (one per cent TBST). Counter staining was done with Harry's Haematoxylin for five min. Blueing of the sections was done in tap water followed by dehydration in ascending grades of isopropyl alcohol. After dehydration and air drying, the sections were cleared in xylene and mounted using Dibutylphthalate polystyrene xylene (DPX) mountant. The mounted slides were viewed under the microscope and representative images recorded. Negative control staining was carried out by substituting the primary antibodies with antibody dilution buffer.

3.5.2.5. Immunohistochemistry scoring *(Vakkala et al., 1999)*

The intensity of the immunostaining with all the antibodies was evaluated by dividing the staining reaction into four groups:-

1 = weak cytoplasmic staining intensity

2 = moderate cytoplasmic staining intensity

3 = strong cytoplasmic staining intensity

4 = very strong cytoplasmic staining intensity.

The quantity of the immunostaining was evaluated as follows:

0 = No positive immunostaining

1 = < 25 per cent of the tumour cells showing cytoplasmic positivity

2 = 25–50 per cent of the tumour cells showing cytoplasmic positivity

3 = 50–75 per cent of the tumour cells showing cytoplasmic positivity

4 = > 75 per cent of the tumour cells showing cytoplasmic positivity.

A combined score for the immunostaining based on both the qualitative and quantitative immunostaining was arrived at, by adding both the qualitative and quantitative score, based on which three groups were categorized :-

\+ = no or weak immunostaining; score 0–2

++ = moderate immunostaining; score 3–5

+++ = strong immunostaining; score 6–8

3.6. STATISTICAL ANALYSIS

The data was analysed statistically using the software SPSS version 24. Fisher's exact test was used to find out association between categorical variables. Wilcoxon signed-rank test was used to compare the means of expression score of the proteins and Spearman's rank correlation was used to find out the relationship between their expressions.

4. RESULTS

4.1. EPIDEMIOLOGY OF CANINE MAMMARY TUMOURS

The occurrence of mammary tumour in dogs was studied on the basis of age, sex, breed and types of tumour. A total of 22 dogs bearing canine mammary growths were studied. Out of these, 20 cases were diagnosed as either benign or malignant mammary tumours and two were identified as non-neoplastic conditions of the mammary gland.

4.1.1. Age-wise occurrence

Age-wise occurrence of canine mammary tumours is presented in Fig. 2.

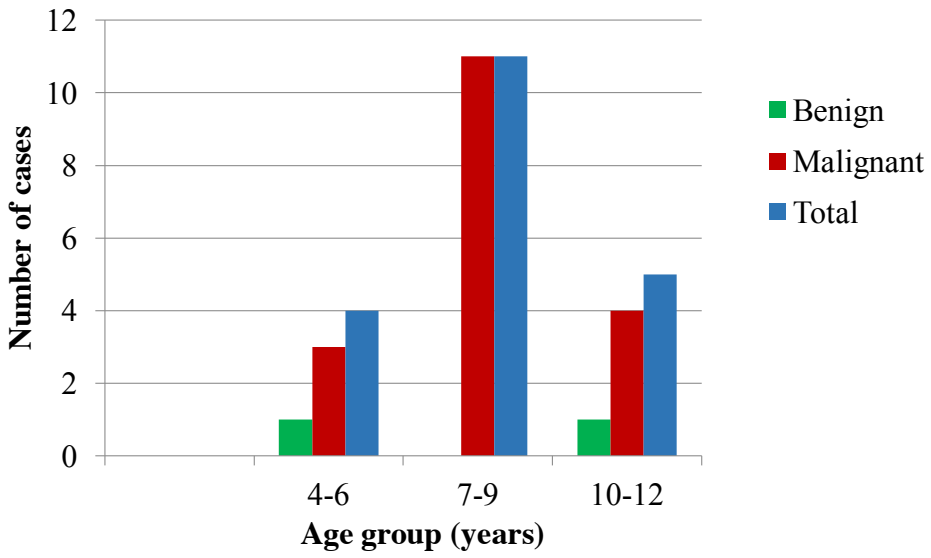

Fig.2. Age-wise occurrence of canine mammary tumours (n=20)

A higher occurrence of mammary tumour was observed in dogs aged between seven and nine years (11/20, 55 per cent) followed by 10-12 years (5/20, 25 per cent) and the least among dogs aged four to six years (4/20, 20 per cent). Out of the 18

malignant mammary tumours, 61 per cent (11/18) were recorded in the seven to nine years age group, 17 per cent (3/18) in the four to six years age group and 22 per cent (4/18) in the 10-12 years group. Of the two benign mammary tumours, one case was observed in the four to six years group and the other in the 10-12 years group.

4.1.2. Gender-wise occurrence

All the cases of CMT were recorded in female dogs.

4.1.3. Breed-wise occurrence

Breed-wise occurrence of CMTs is shown in Fig. 3.

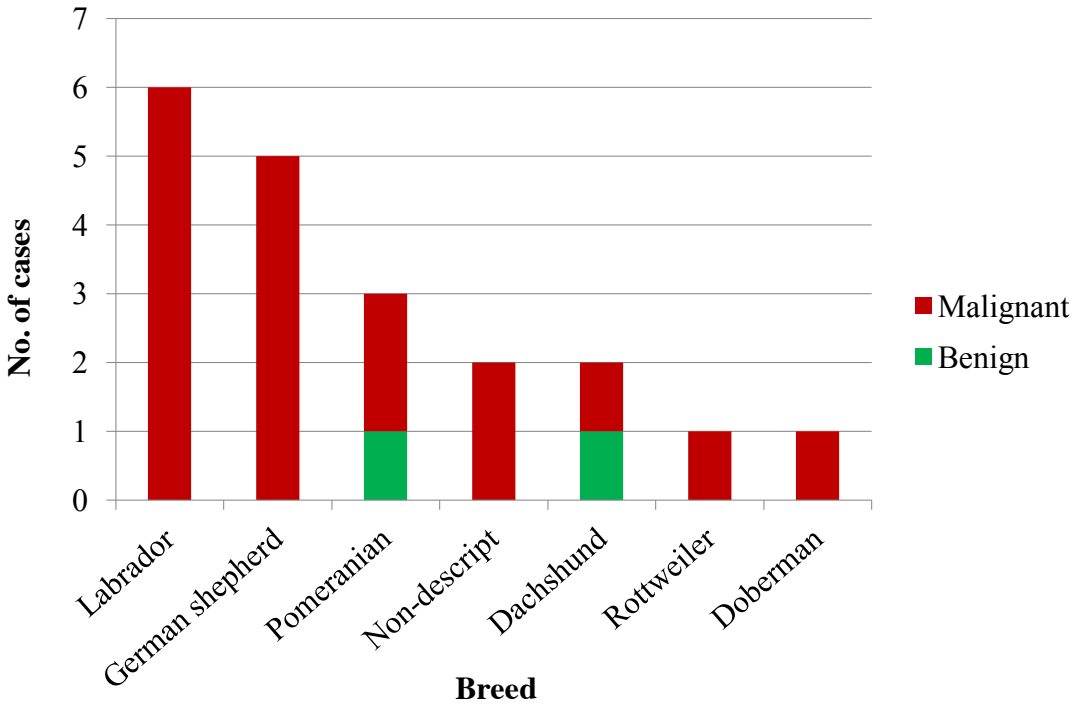

Fig. 3. Breed-wise occurrence of canine mammary tumours

Occurrence of mammary tumours was seen slightly more in Labrador (6/20, 30 per cent) followed by German shepherd (5/20, 25 per cent), Pomeranian (3/20, 15

per cent), Non-descript (2/20, 10 per cent), Dachshund (2/20, 10 per cent) and solitary cases in Rottweiler and Doberman.

4.1.4. Tumour-wise occurrence

Occurrence of different types of mammary tumour is shown in Fig. 4. Among the different types, ductal carcinoma (DC) was the most frequent type (45 percent) followed by carcinoma arising in benign mixed tumour (CABMT) (20 percent), comedocarcinoma (10 per cent) and fibroadenoma (10 per cent). One case each of spindle carcinoma, tubulopapillary carcinoma (TP) and solid carcinoma were also identified.

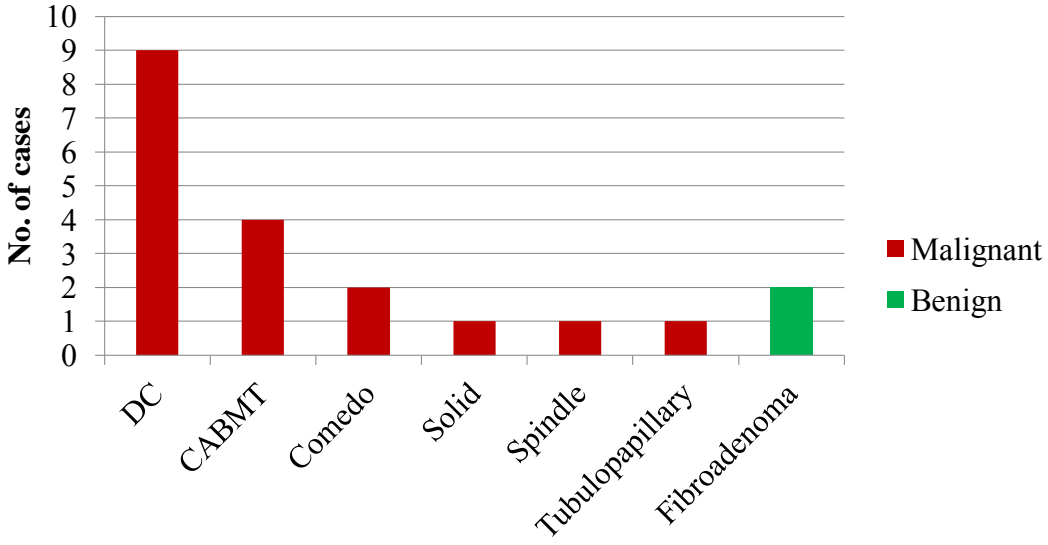

Fig. 4. Tumour-wise incidence of canine mammary tumours (n=20)

4.2. GROSS PATHOLOGY OF CANINE MAMMARY TUMOURS

Grossly most of the tumours were grayish to yellowish white in colour (Plate 1 to 4). Majority of the mammary tumours were observed in the caudal glands viz abdominal and inguinal mammary glands. The size of the mammary tumours ranged between one cm to 13 cm with the mean size of 6.70±0.60 cm. Majority of the CMTs were soft to firm in consistency except four cases which were hard to cut. The gross pathological features observed in the study are tabulated in Table 1.

Table 1.Gross pathology of canine mammary tumours

Case No.	Breed	Sex	Age (Years)	Gland affected	Size (cm)	Shape	Consistency	Colour
1	Labrador	F	8	Caudal abdominal	10	Elliptical	Soft	Greyish white
2	German shepherd	F	10	Caudal abdominal	8	Irregular	Soft	Greyish white
3	Labrador	F	8	Caudal thoracic	7	Ovoid	Soft	Greyish white
4	Non-descript	F	10	Cranial abdominal	8	Irregular	Firm	Greyish white
5	Rottweiler	F	10	Caudal abdominal	13	Round	Soft	Red
6	Labrador	F	10	Caudal thoracic	3	Round	Soft	Greyish White
7	Doberman	F	4	Cranial abdominal	1	Nodular	Firm	Greyish White
8	German shepherd	F	9	Caudal abdominal	7	Irregular	Soft	Greyish White
9	Labrador	F	8	Caudal thoracic	8	Round	Soft	Greyish White
10	German shepherd	F	8	Caudal thoracic	8	Round	Soft	Yellowish
11	Pomeranian	F	9	Inguinal	5	Irregular	Firm	Reddish white
12	Labrador	F	7	Caudal abdominal	8	Round	Firm	Greyish White
13	German shepherd	F	7	Inguinal	6	Irregular	Firm	Reddish white

Case No.	Breed	Sex	Age (Years)	Gland affected	Size (cm)	Shape	Consistency	Colour
14	Labrador	F	8	Cranial abdominal	8	Ovoid	Firm	Reddish white
15	Non-descript	F	5	Caudal abdominal	6	Irregular	Hard	Greyish White
16	Dachshund	F	5	Caudal abdominal	2	Elliptical	Hard	Greyish White
17	Pomeranian	F	8	Inguinal	7	Irregular	Hard	Greyish White
18	German shepherd	F	7	Caudal abdominal	8	Irregular	Hard	Greyish White
19	Pomeranian	F	10	Cranial abdominal	5	Irregular	Firm	Reddish white
20	Dachshund	F	6	Inguinal	6	Round	Firm	Yellowish white

4.3. HISTOPATHOLOGY OF CANINE MAMMARY GROWTHS

Based on histopathology the 22 cases were categorised as neoplastic (n=20) and non-neoplastic (n=2) growths. Of the 20 neoplastic conditions, 18 were identified as malignant (Table 2) and two benign as (Table 3). The non-neoplastic growths identified are tabulated in Table 4.

Table 2. Malignant canine mammary tumours diagnosed

Sl.No.	Tumour type	Total
1	Ductal carcinoma	9
2	Carcinoma arising in benign mixed tumour	4
3	Comedocarcinoma	2
4	Spindle cell carcinoma	1
5	Tubulopapillary carcinoma	1
6	Solid carcinoma	1
	Total	18

Table 3. Benign canine mammary tumours diagnosed

Sl.No.	Tumour type	Total
1	Fibroadenoma	2
	Total	2

Table 4. Non-neoplastic mammary lesions diagnosed

Sl.No.	Tumour type	Total
1	Lobular hyperplasia	1
2	Lactational adenosis	1
	Total	2

Ductal carcinomas were composed of groups of pleomorphic, polyhedral to oval cells with hyperchromatic nuclei. The cells were arranged in groups or sheets in an attempted glandular pattern. Fibrous tissue proliferation around the glandular region and infiltration of inflammatory cells could be seen (Plate 5).

Solid carcinoma consisted of sheets of cells without lumina. The cells were oval, with scant cytoplasm and poorly demarcated cell margins. Nuclei were

hyperchromatic and oval with a single central basophilic nucleolus. Moderate anisocytosis, anisokaryosis and variable number of mitoses could be noticed (Plate 6).

In comedocarcinomas, the necrotic areas in the centre of neoplastic cell aggregates were characteristic. The neoplastic cells were arranged in sheets with fine fibrovascular stroma without any tubular differentiation. The necrotic centre contained amorphous and eosinophilic material with cell debris, macrophages and neutrophils (Plate 7).

In the spindle cell variant carcinoma, the neoplastic cells were predominantly spindle shaped. Majority of the cells and nuclei were large and fusiform with moderate pleomorphism. Cords and islands of neoplastic cells with fibrovascular stroma surrounding it could be seen (Plate 8).

Tubulopapillary carcinoma was characterised by proliferation of the epithelial cells in a pedunculated and papillary fashion. The papillae were supported by fine fibrovascular connective tissue stalks which were found extending into the tubular lumina (Plate 9).

Carcinomas arising in benign mixed tumour (CABMT) were composed of both benign and malignant components. The chondroid component was well differentiated comprised of fibroblasts, collagen and cartilage. However, multifocal clusters of hyperchromatic epithelial cells with marked nuclear and cellular pleomorphism (foci of carcinoma) could be noticed (Plate 10).

Microscopically, fibroadenoma consisted of proliferating well-differentiated ducts lined by abundant loose fibrovascular stroma. The ductal epithelium was composed of uni or bilayered cuboidal to columnar epithelial cells. Mitotic figures were rare. The elongated stromal cells which had oval nuclei were embedded into the eosinophilic extracellular matrix (Plate 11).

Lobular hyperplasia exhibited non-neoplastic proliferation of ducts and acini. The epithelial cells showed mild epithelial hyperplasia, nuclear hyperchromasia and mild anisocytosis and anisokaryosis.

4.4. GRADING OF CANINE MAMMARY TUMOURS

The malignant tumours were graded according to Clemente *et al*., 2010. Histological malignancy grade wise classification of the malignant mammary tumours is given in Fig. 5. Of the 18 malignant mammary tumours six (34 per cent) were grade I, eight (44 per cent) were grade II and four (22 per cent) were grade III. The tumour-wise histological grading is represented in Fig. 6. It was observed that majority of the simple carcinomas, (12 out of 14) which comprised of ductal carcinoma, solid carcinoma, comedocarcinoma, spindle carcinoma and tubulopapillary carcinoma were either grade II or III where as all the mixed tumours (CABMT) were classified as grade II. Relationship between the histological grading and tumour type is shown in Fig. 7.

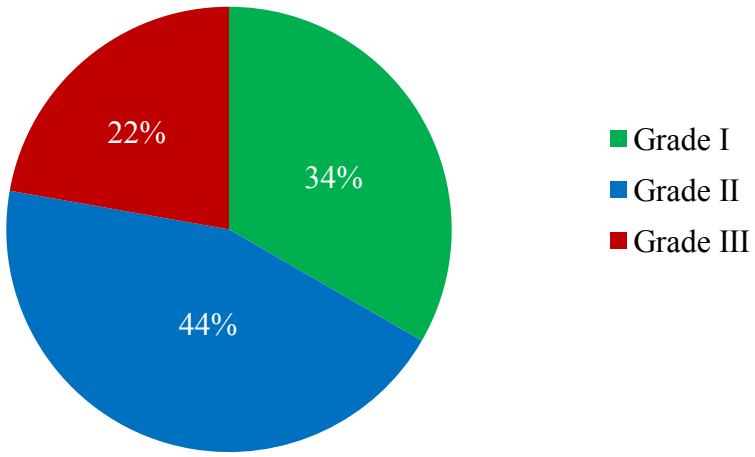

Fig.5. Grade-wise occurrence of malignant mammary tumours

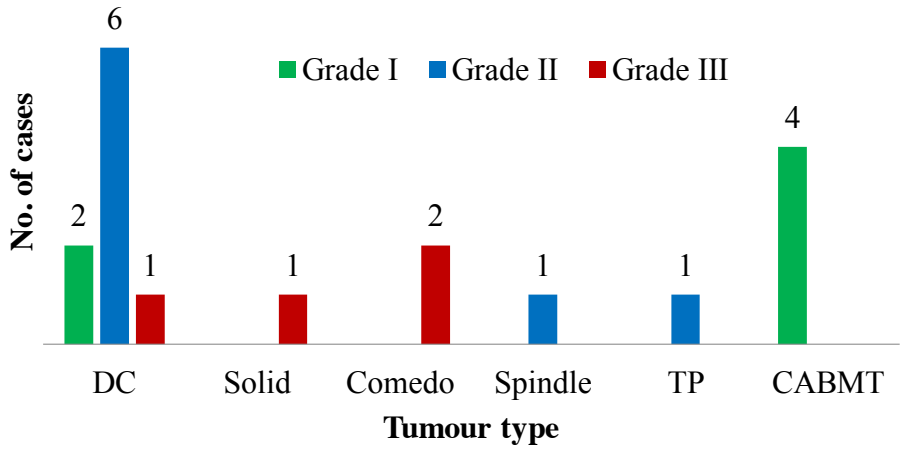

Fig. 6. Tumour-wise grading of canine mammary tumours. DC: Ductal carcinoma; TP: Tubulopapillary carcinoma; CABMT: Carcinoma arising in benign mixed tumour

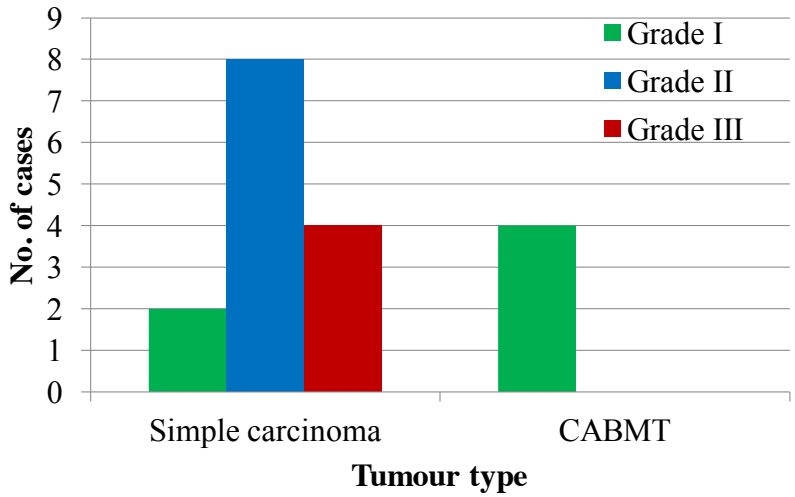

Fig.7. Relationship between the tumour type and histological grading

4.5. EPIDEMIOLOGY OF CANINE SUPERFICIAL TUMOURS

A total of 17 superficial tumours were collected during the study and were examined on the basis of the age, sex, breed and type of the tumour.

4.5.1. Age-wise occurrence

Age-wise occurrence of canine superficial tumours is presented in Fig 8. The age group of seven to nine years showed highest incidence (47.06 per cent) followed by four to six years (29.41 per cent), 10-12 years (11.76 per cent) and less than three years (11.76 per cent).

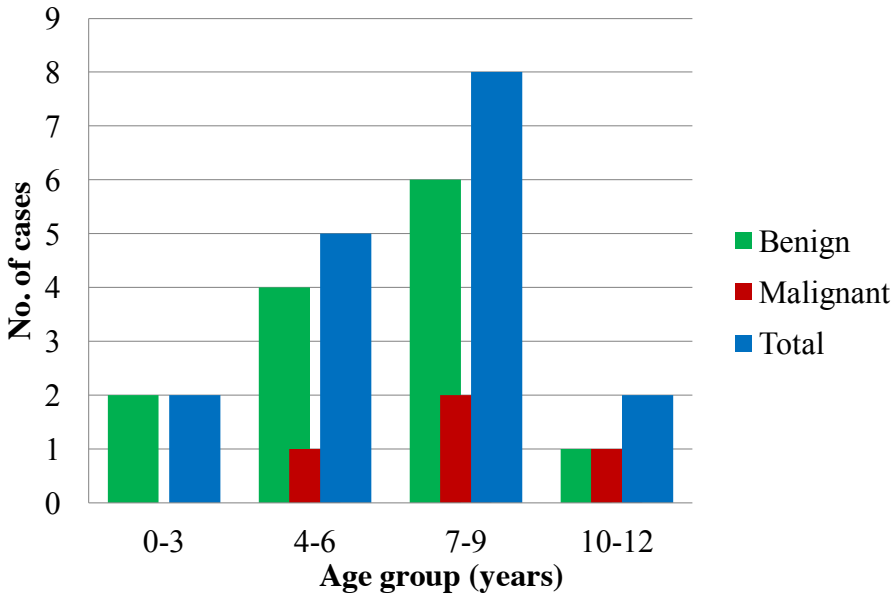

Fig. 8. Age-wise occurrence of canine superficial tumours

4.5.2. Gender-wise occurrence

Gender-wise occurrence of canine superficial tumours is presented in Fig. 9. It is seen that the occurrence of superficial tumours is slightly more in male dogs (58.82 per cent) than females (41.18 per cent). There was no association between the sex and occurrence of superficial tumours ($p>0.05$). Among the conditions recorded,

sebaceous adenoma, myxosarcoma, hepatoid adenoma, squamous cell carcinoma and haemangioma were seen in male dogs where as fibroma, trichoepithelioma, melanoma and fibrosarcoma were reported in female dogs. Lipoma and trichoblastoma were observed in both males and females.

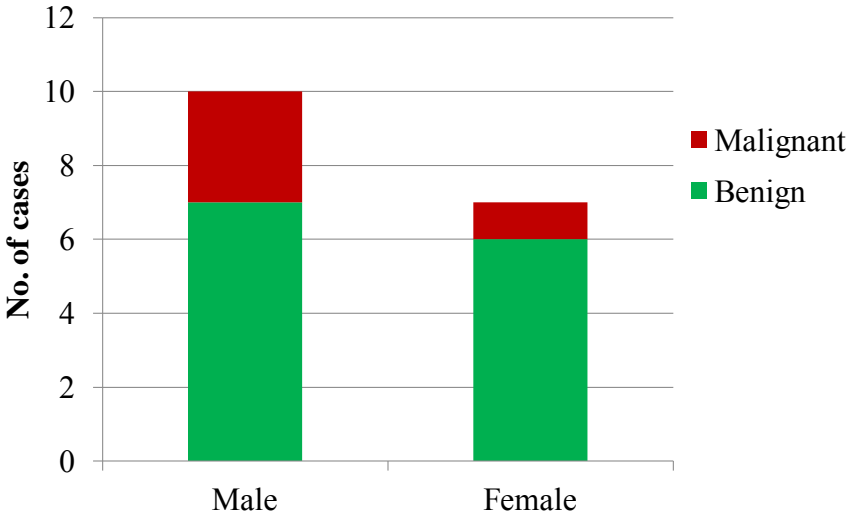

Fig. 9. Gender-wise occurrence of canine superficial tumours

4.5.3. Breed-wise occurrence

Breed-wise occurrence of canine superficial tumours is presented in Fig. 10. The incidence was seen more in Labradors (41.18 per cent) followed by Rottweiler (17.65 per cent), Non-descript (11.76 per cent), German shepherd (11.76 per cent) and Dachshund (11.76 per cent). One case was seen in a cross-bred (CB) dog.

4.5.4. Tumour-wise occurrence

Among the samples analysed in the present study, 76.47 percent (13 out of 17) were benign tumours and rest 23.53 percent (4 cases) were malignant counterparts. The tumour-wise occurrence of canine superficial tumours observed in the present study is shown in Fig. 11.

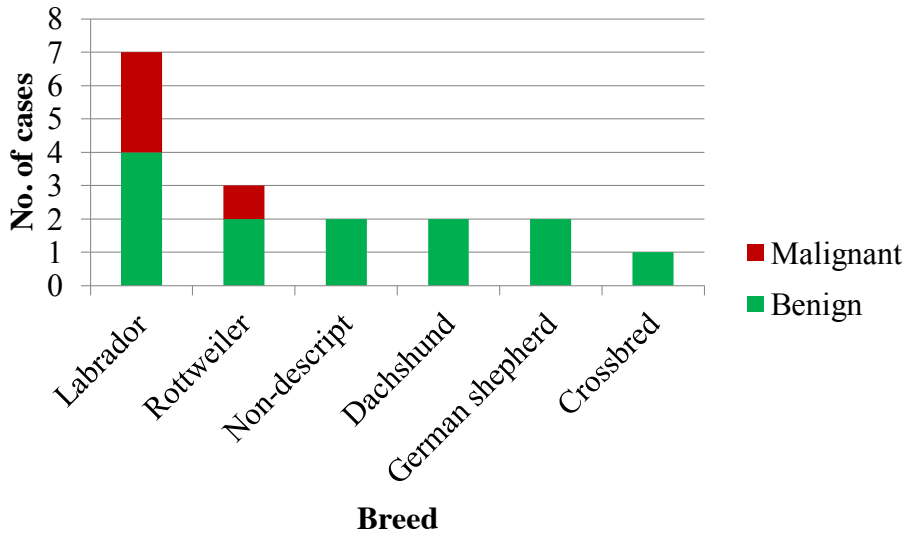

Fig. 10. Breed-wise occurrence of canine superficial tumours

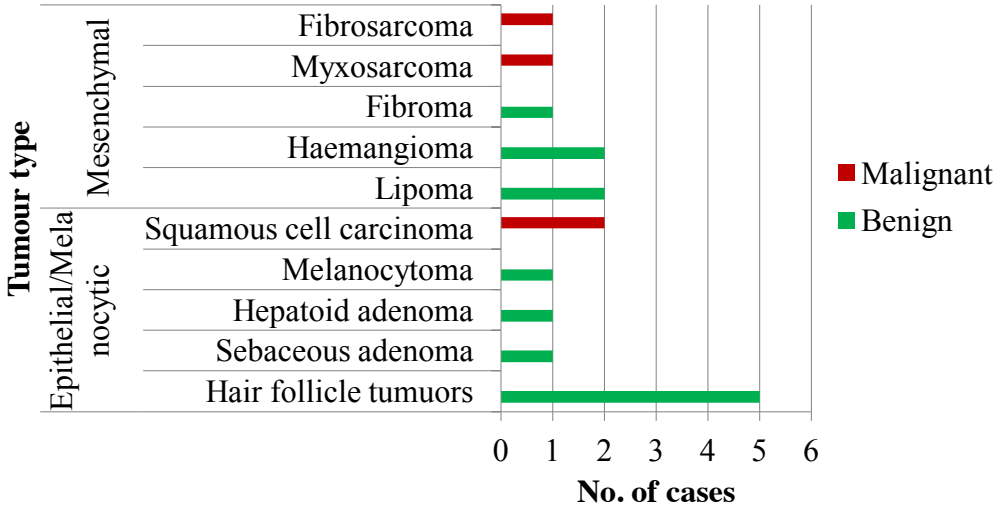

Fig. 11. Tumour-wise occurrence of canine superficial tumours

4.6. GROSS PATHOLOGY OF CANINE SUPERFICIAL TUMOURS

Grossly, majority of the tumours were round to oval in shape with soft to firm consistency and grayish white colour (Plate 12 to 16). The gross pathological features of the tumours observed during the study are presented in Table 5.

Table 5. Gross pathology of canine superficial tumours

Case. No	Breed	Sex	Age (Yrs)	Location	Shape	Size (cm)	Consistency	Colour	Diagnosis
1	Dachshund	M	2	Abdomen	Oval	7	Firm	Greyish white	Sebaceous adenoma
2	Rottweiler	F	5	Right thigh	Round	3	Firm	Greyish brown	Fibroma
3	Labrador	M	8	Left thigh	Irregular	6	Firm	Reddish white	Myxosarcoma
4	Labrador	F	9	Dorsum	Round	4	Soft	Greyish white	Trichoepithelioma
5	Labrador	M	8	Forehead	Round	8	Firm	Greyish white	Trichoblastoma
6	Crossbred	M	8	Abdomen	Round	4	Soft	White	Lipoma
7	Dachshund	M	4	Perineum	Irregular	4	Soft	Greyish white	Hepatoid adenoma
8	Rottweiler	F	4	Left forelimb digit	Round	3	Firm	Black	Melanocytoma
9	Rottweiler	M	6	Left forelimb digit	Irregular	3	Hard	Greyish white	Squamous cell carcinoma
10	Non-descript	M	3	Neck	Round	5	Firm	Greyish white	Trichoblastoma
11	Labrador	F	10	Vulva	Round	5	Hard	White	Fibrosarcoma
12	Labrador	F	5	Abdomen	Round	7	Soft	White	Lipoma
13	German shepherd	F	7	Forehead	Round	5	Firm	Greyish white	Trichoblastoma
14	Non-descript	M	10	Left thigh	Oval	3	Firm	Red	Haemangioma
15	Labrador	M	7	Eyelid	Round	4	Firm	White	Squamous cell carcinoma
16	German shepherd	F	9	Abdomen	Round	5	Soft	Greyish white	Trichoepithelioma
17	Labrador	M	7	Abdomen	Oval	3	Soft	Red	Haemangioma

4.7. HISTOPATHOLOGY OF CANINE SUPERFICIAL TUMOURS

Histopathology of the tumours revealed 13 benign (Table 6) and four malignant tumours (Table 7). It was also found that majority of the neoplasms (59 per cent) were epithelial/melanocytic in origin (Fig. 11).

Table 6. Histopathological classification of benign superficial tumours

Sl. No.	Tumour type	No. of cases
1	Trichoblastoma-Ribbon type	3
2	Lipoma	2
3	Sebaceous adenoma	1
4	Fibroma	1
5	Trichoepithelioma-Cystic variant	2
6	Hepatoid adenoma	1
7	Melanocytoma	1
8	Haemangioma	2
	Total	13

Table 7. Histopathological classification of malignant superficial tumours

Sl. No.	Tumour type	No of cases
1	Myxosarcoma	1
2	Squamous cell carcinoma	2
3	Fibrosarcoma	1
	Total	4

Trichoblastomas (three cases) were multilobulated and consisted of ribbon type cords of branching and anastamosing cells. The cords had two or three cells thickness. The neoplastic cells showed a palisaded appearance with scant cytoplasm and hyperchromatic nuclei with inconspicuous nucleoli. Variable numbers of mitotic figures were also seen. Moderate quantity of stroma was observed between the cords of cells (Plate 17).

Two cases of lipoma recorded in the abdominal region. They were characterised by adipocytes that had vacuolated cytoplasm and peripherally compressed nuclei. The lipocytes infiltrated in between the muscle fibers and collagen thick connective tissue. Few infiltrating neutrophils also could be seen. (Plate 18).

A solitary case of sebaceous adenoma revealed mature sebocytes, with pale and abundant vacuolated eosinophilic cytoplasm and centrally placed hyperchromatic nuclei arranged in multiple lobules and separated by connective tissue trabeculae. The margin of the lobules had multilayered basophilic reserve cells with prominent hyperchromatic nuclei and scant cytoplasm. Occasional mitoses could be observed (Plate 19).

Histological features of fibroma was characterised by proliferation of mature fibrocytes producing abundant collagen. The collagen fibers were arranged in interwoven fascicles. The neoplastic fibrocytes were low in number compared with the abundant collagen. The nuclei of fibrocytes were oval, normochromatic and the cytoplasm was indistinct which blended into the extracellular collagenous stroma (Plate 20).

In the cystic variant of trichoepithelioma (two cases), several small and large cysts filled with keratinous debris were seen. The basal lamina was thickened and eosinophilic, with palisaded cells having scant cytoplasm and hyperchromatic nuclei. Towards the centre of the cyst, the cells were more haphazardly arranged. The cystic lumen consisted of shadow (ghost) cells and keratinous debris (Plate 21).

Hepatoid gland adenoma was characterised by proliferation of cells that were arranged in cords and anastomosing trabeculae that had morphological similarity to normal hepatocytes. The cells were polyhedral in shape with centrally placed, large ovoid, vesicular and normochromatic nuclei. The cytoplasm was eosinophilic and the borders were distinct. The periphery of the lobules had single layer of basaloid reserve cells, with hyperchromatic nuclei and scant cytoplasm (Plate 22).

A solitary case of melanocytoma recorded in the digit was characterised by abundance of melanin pigment. The proliferating melanocytes, arranged singly and in clusters, had large quantity of intra-cytoplasmic melanin which obscured the nuclear morphology (Plate 23).

On histological examination, haemangiomas were identified as cavernous types which consisted of varying sized vascular spaces filled with erythrocytes and lined by single layer of endothelial cells whose nuclei were inconspicuous. Organized thrombi with foci of haemosiderosis were found in the tumour. Fibrous connective tissue stroma with infiltrated inflammatory cells was present between the vascular channels (Plate 24).

Microscopical examination of myxosarcoma revealed proliferation of spindle shaped fibroblasts, arranged loosely, in an abundant myxoid matrix which stained light blue. Increased cellularity, nuclear density and pleomorphism were observed (Plate 25).

Out of the two cases of squamous cell carcinoma (SCC), one showed trabeculae and cords of neoplastic squamous epithelial cells which invaded the dermis and subcutis. The neoplasm was well differentiated with extensive keratosis and formation of distinct keratin pearls (Plate 26). The second case, which was less differentiated, had proliferation of neoplastic squamous epithelial cells in cords with minimal keratinisation (Plate 27).

In a solitary case of fibrosarcoma recorded in the vulva, the neoplasm was well differentiated, with spindle shaped tumour cells arranged in herringbone pattern. Cytoplasm was scant and nuclei were oval to elongated with inconspicuous nucleoli (Plate 28). Few multinucleated giant cells were seen, which had oval nuclei and prominent nucleoli.

4.8. IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed to study the expression of mTOR and DEPTOR in all the cases of canine mammary and superficial tumours.

4.8.1. Expression of Mechanistic target of Rapamycin (mTOR) in canine mammary tumours

Immunostaining pattern of mTOR in various subtypes of CMTs is given in Fig. 12. All the tumours had higher expression of mTOR when compared to the normal mammary gland ($p<0.01$). mTOR immunostaining was observed in the cytoplasm of the neoplastic epithelial cells. Forty per cent (8/20) of the tumours were strongly positive (+++) and 45 per cent (9/20) were moderately positive (++). Rest 15 per cent (3 cases) have shown weak immunostaining (+). Except one case of ductal carcinoma, all the malignant tumours showed strong to moderate expression of mTOR. The two benign cases (fibroadenoma) showed weak expression. The variation in staining reaction for mTOR in different cases of CMT is shown in Plates 29 to 37.

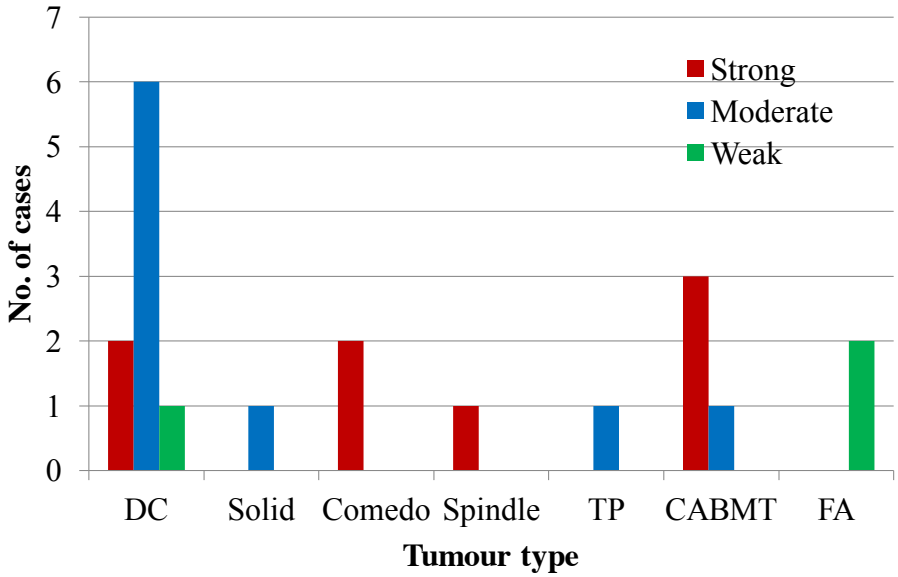

Fig.12. Expression of mTOR in canine mammary tumours. DC: Ductal carcinoma; TP: Tubulopapillary carcinoma; CABMT: Carcinoma arising in benign mixed tumour; FA: Fibroadenoma

4.8.1.1. Relationship between age groups of animals and **mTOR** *expression*

Expression of mTOR based on the age group of the animals is shown in Fig.13. Strong expression of mTOR was observed in 50 percent and 55 per cent of cases in the dogs aged four to six years and seven to nine years respectively. Rest of the cases in the above age groups and all the cases in the 10-12 years age group have shown moderate to weak expression. On statistical analysis, it was found that there was no correlation between the age of the animals and mTOR expression (Spearman's correlation coefficient r_s= -0.27, p=0.25).

4.8.1.2. Relationship between **mTOR** *expression and tumour grade*

Expression of mTOR in malignant mammary tumours of various grades is shown in Fig. 14. Analysis of the data revealed that there was poor correlation between the grade of the tumour and mTOR expression (rs= 0.231, p= 0.328).

4.8.1.3. Relationship between **mTOR** *expression and size of mammary tumours*

Expression of mTOR on the basis of the tumour size is represented in Fig. 15. The correlation between the size of the tumours and mTOR expression was poor (rs= 0.006, p= 0.980).

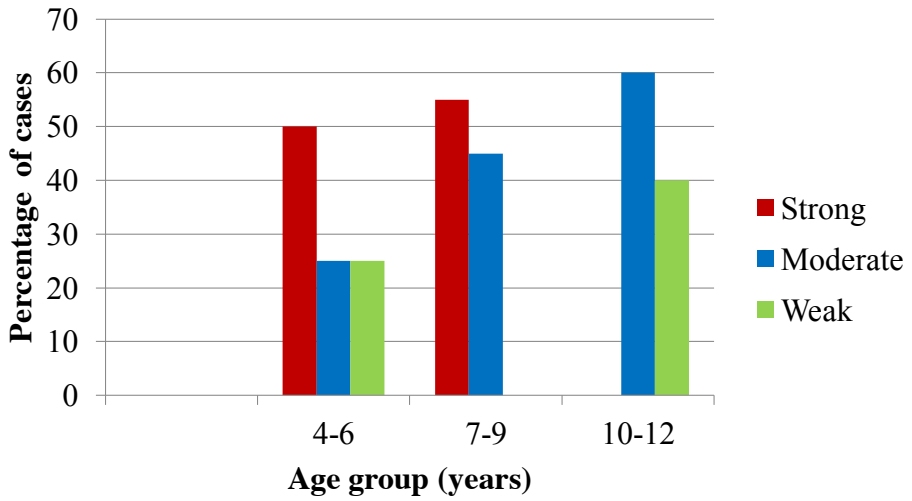

Fig.13.Expression of mTOR in different age groups of dogs with mammary tumours

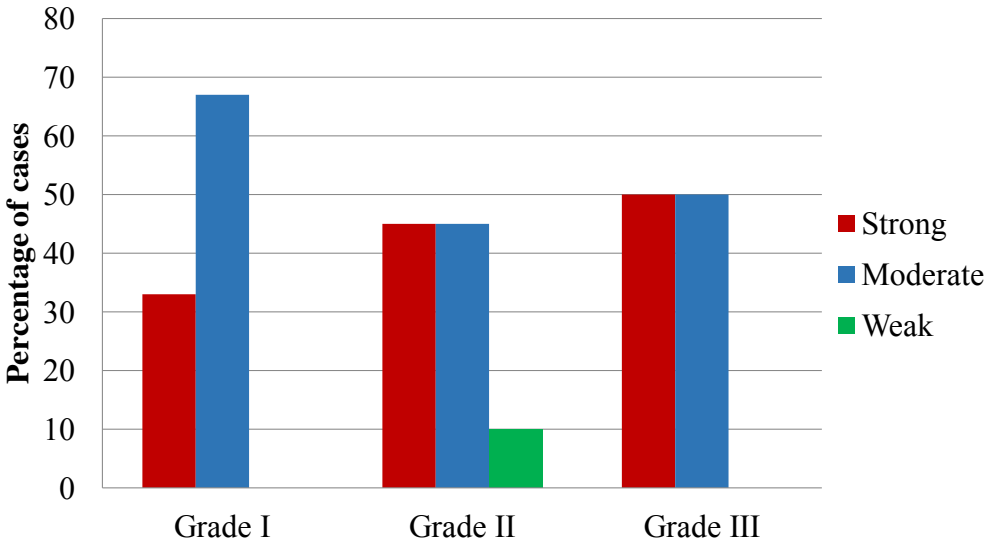

Fig.14. Expression of mTOR in various grades of malignant mammary tumours

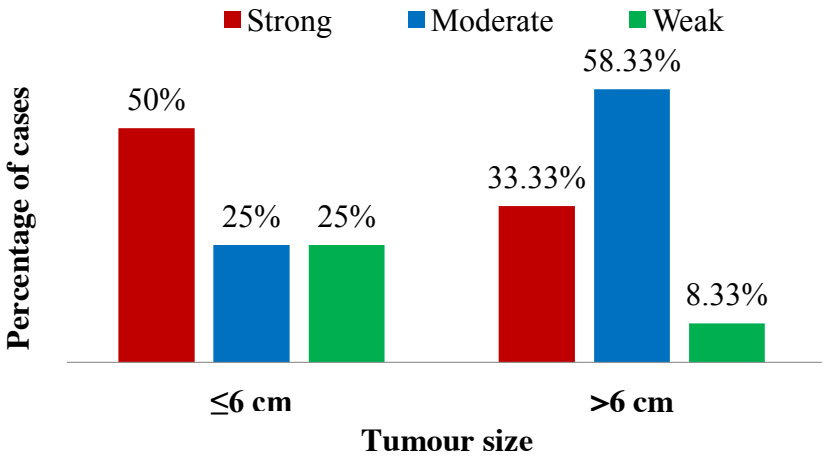

Fig.15. Expression of mTOR based on tumour size

4.8.2. Expression of DEPTOR in CMTs

Expression pattern of DEPTOR in canine mammary tumours is given in Fig. 16. The immunolabelling was observed mainly in the cytoplasm of the neoplastic

epithelial cells. Strong expression of DEPTOR was recorded in only one case of CABMT, in the glandular component. In 70 per cent (14/20) of the cases moderate expression of DEPTOR was observed. Weak DEPTOR immunostaining was recorded in five cases. All the malignant tumours except three cases of ductal carcinoma showed moderate to strong expression of DEPTOR whereas the two benign tumours showed weak expression of DEPTOR (Plate 38 to 43).

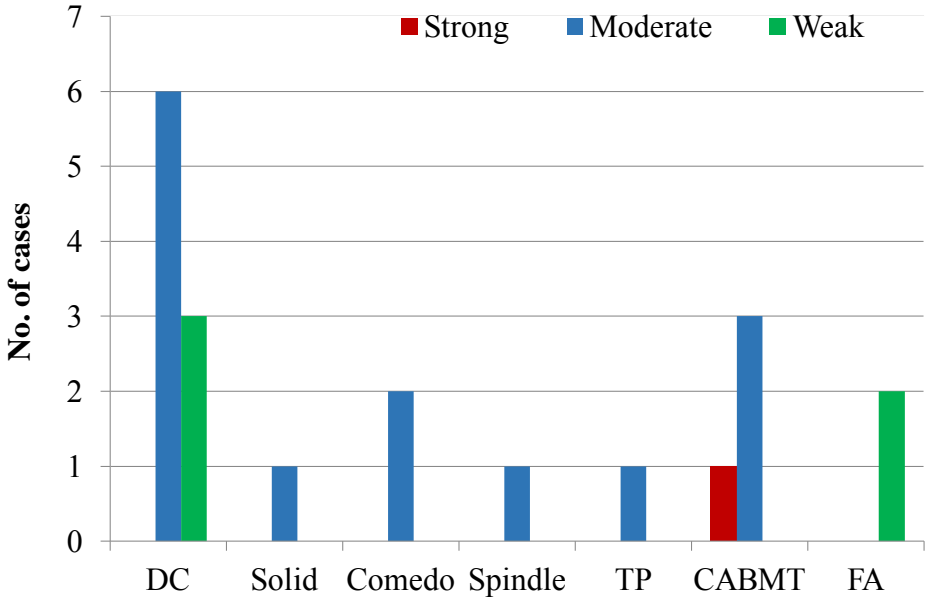

Fig.16. Immunoexpression of DEPTOR in canine mammary tumours. DC: Ductal carcinoma; TP: Tubulopapillary carcinoma; CABMT: Carcinoma arising in benign mixed tumour; FA: Fibroadenoma

4.8.2.1. Relationship between age group of the animals and expression of DEPTOR

Expression of DEPTOR in CMTs on the basis of the age group of the animals is given in Fig. 17. Twenty five per cent of the cases in the four to six year age group have shown strong immunolabelling. Moderate to weak expression was observed in all the remaining cases in all the age groups. Statistical analysis revealed that there

was poor correlation between the animals' age and DEPTOR expression (r_s= 0.258, p= 0.271).

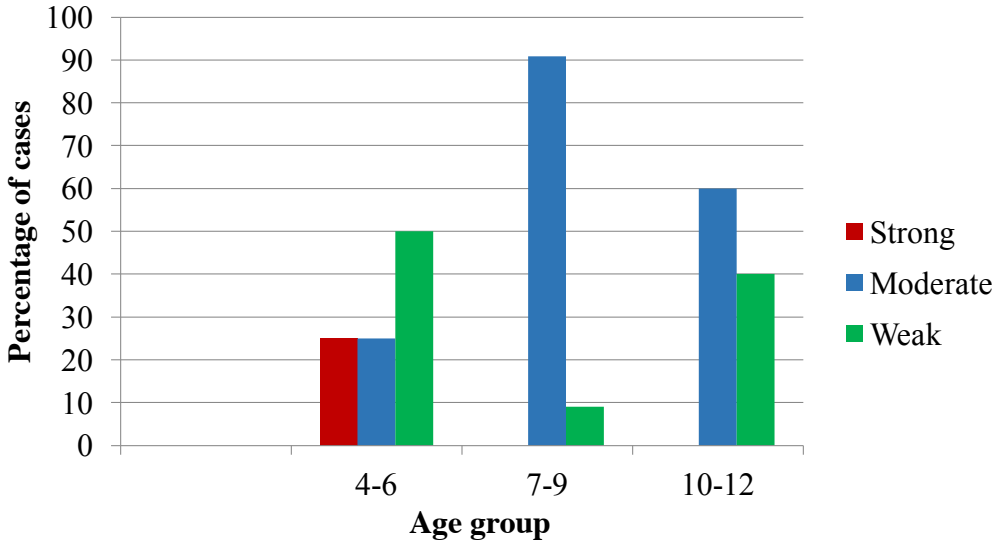

Fig.17. Expression of DEPTOR in different age groups of dogs bearing mammary tumours

4.8.2.2. Relationship between **DEPTOR** *expression and tumour grade*

Summary of the relationship between the grading and immunoscoring of DEPTOR expression in case of malignant mammary tumours is given in Fig. 18. Analysis of the data revealed that there was no significant correlation between the grade of the tumour and DEPTOR expression (r_s= 0.256, p= 0.275).

4.8.2.3. Relationship between **DEPTOR** *expression and size of mammary tumours*

Percentage of cases that showed strong, moderate and weak expression based on the tumour size is depicted in Fig. 19. There was no significant correlation between the tumour size and DEPTOR expression (r_s= -0.124, p= 0.601).

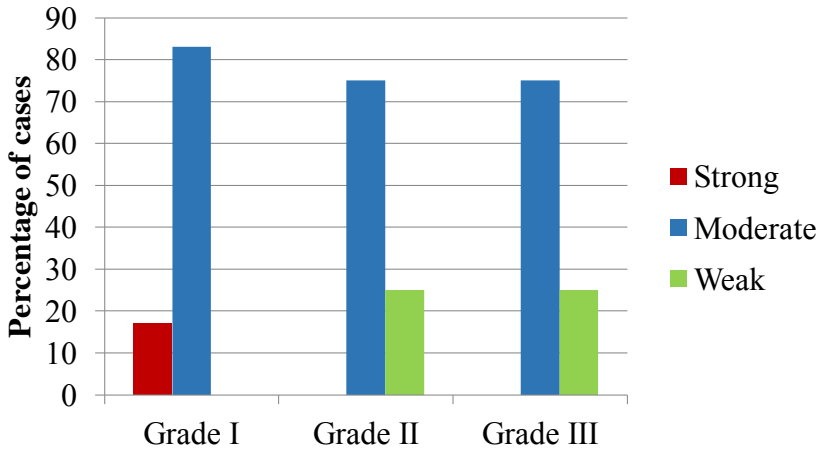

Fig.18. Expression of DEPTOR in various grades of malignant mammary tumours

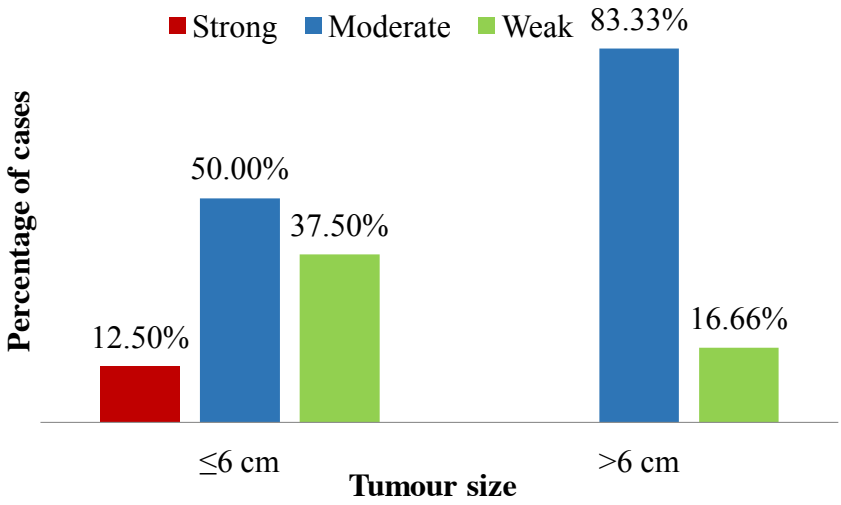

Fig.19. Tumour size-wise expression of DEPTOR in CMTs

4.8.2.4. Relationship between **mTOR** *and* **DEPTOR** *expression in mammary tumours*

Relative expression score of mTOR and DEPTOR in various subtypes of canine mammary tumour is shown in Fig. 20. It was seen that majority of the cases (15 out of 20) have shown strong/moderate expression for both mTOR and DEPTOR. The mean score of expression for mTOR (4.85±0.38) was significantly higher ($p<0.01$) than that of DEPTOR (3.85±0.28). Also there was significant positive correlation (Fig. 21) between expression of mTOR and DEPTOR in canine mammary tumours ($r_s= 0.665$, $p<0.01$).

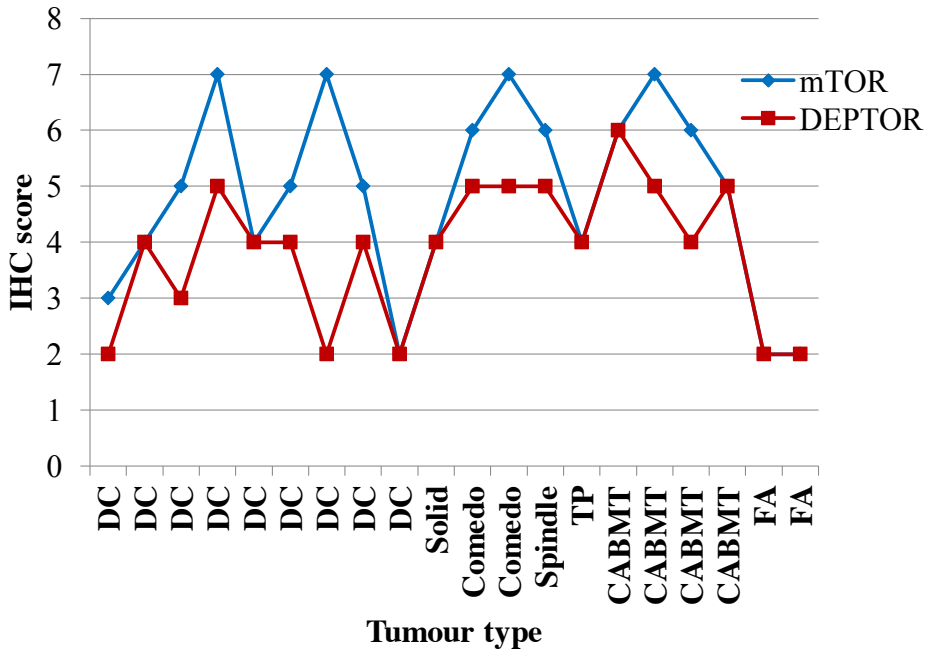

Fig. 20. Relative expression of mTOR and DEPTOR in CMTs. DC: Ductal carcinoma; TP: Tubulopapillary carcinoma; CABMT: Carcinoma arising in benign mixed tumour; FA: Fibroadenoma

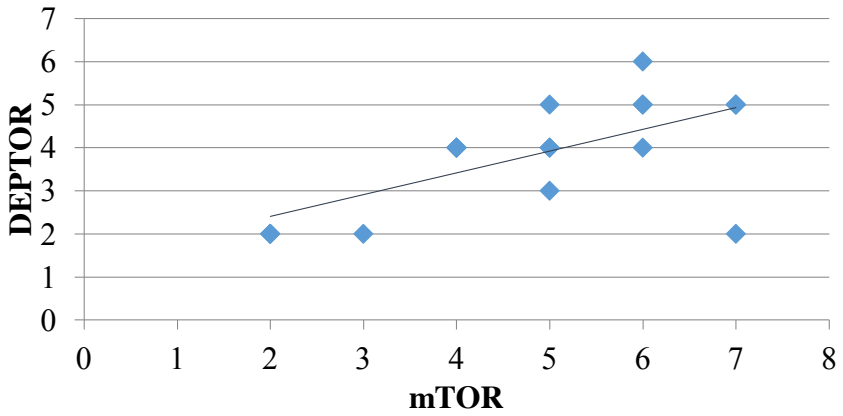

Fig. 21. Scatter plot showing positive correlation between expression score of mTOR and DEPTOR in CMTs

4.8.3. Expression of mTOR in canine superficial tumours

Expression pattern of mTOR in various canine superficial tumours (CSTs) identified in the present study is given in Fig. 22. Seven out of seventeen (41.18 percent) cases showed strong positivity (+++) for mTOR immunostaining and 29.41 per cent (five out of 17) of the tumours were moderately positive (++). Rest 29.41 per cent (five cases) has shown weak immunostaining (+) (Plate 44 to 52).

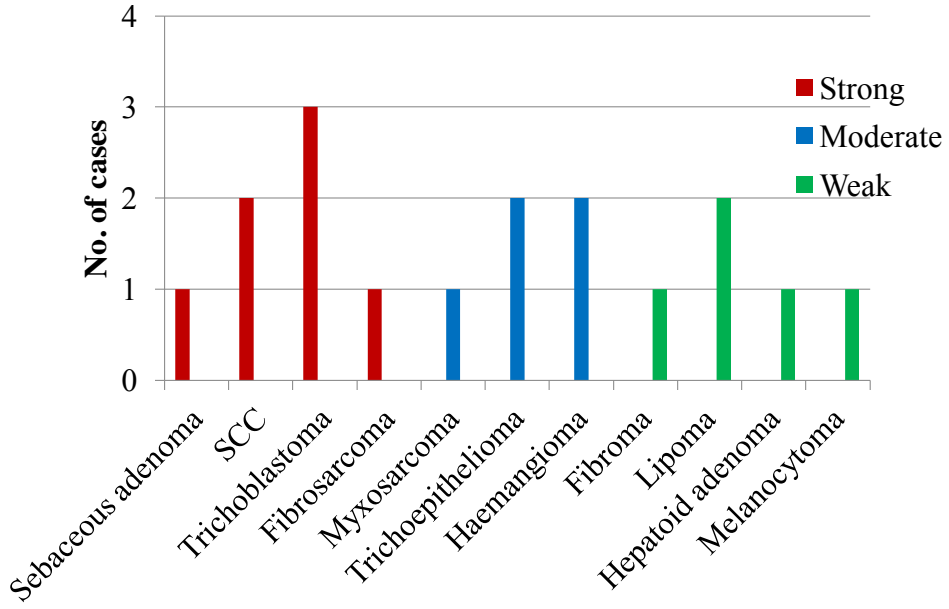

Fig.22. Expression of mTOR in canine superficial tumours

4.8.3.1. Relationship between **mTOR** *expression and age of the animals*

Age-wise mTOR expression in CSTs is given in Fig. 23. On statistical analysis, it was found that there was poor correlation between the age of the animals and mTOR expression in case of CSTs (r_s= 0.177, p= 0.498).

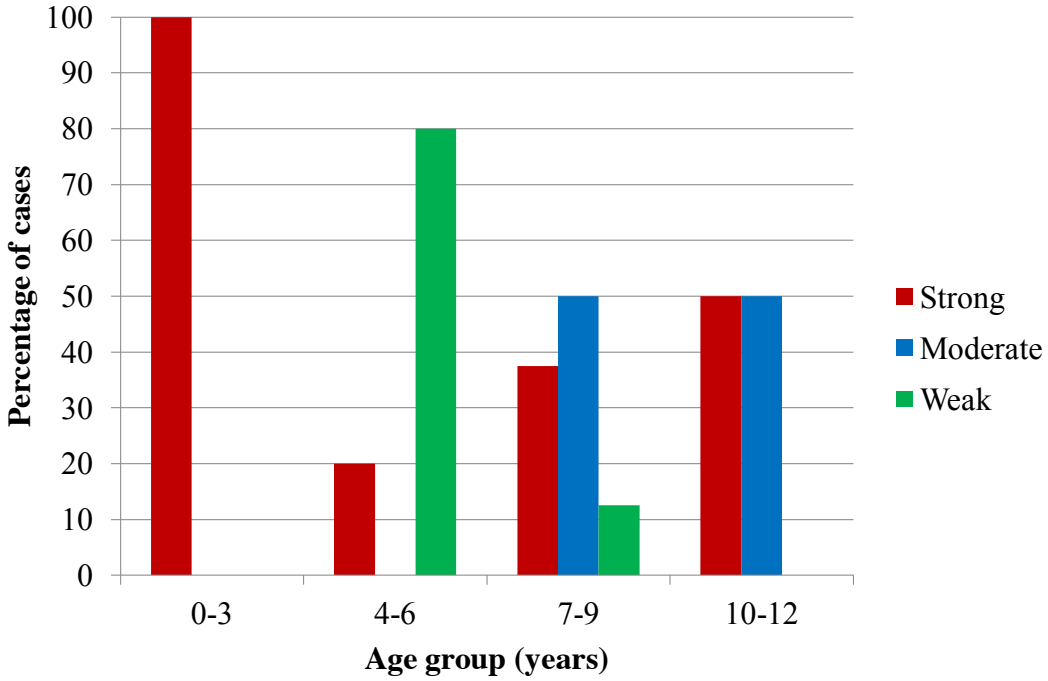

Fig. 23. Expression of mTOR in various age groups of dogs bearing superficial tumours

4.8.3.2. Relationship between **mTOR** *expression and size of superficial tumours*

Percentage of cases that showed strong, moderate and weak expression of mTOR based on the size of the tumours is given in Fig. 24. There was poor correlation between the size of the tumours and expression of mTOR (rs= 0.441, p= 0.077).

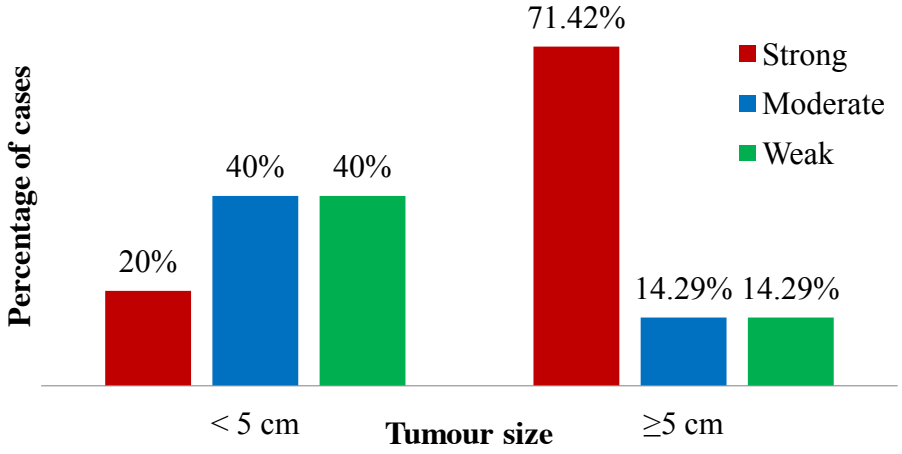

Fig.24. Expression of mTOR based on tumour size in canine superficial tumours

4.8.4. Expression of DEPTOR in canine superficial tumours

Expression pattern of DEPTOR in CSTs is given in Fig. 25. In none of the cases DEPTOR was strongly expressed. Seven cases (41.18 per cent) showed moderate expression and the remaining 10 cases (58.82) revealed weak expression. The immunolabelling was localized to the cytoplasm of the neoplastic epithelial cells. The variation in staining reaction for DEPTOR in different cases of CST is shown in Plate 53 to 60.

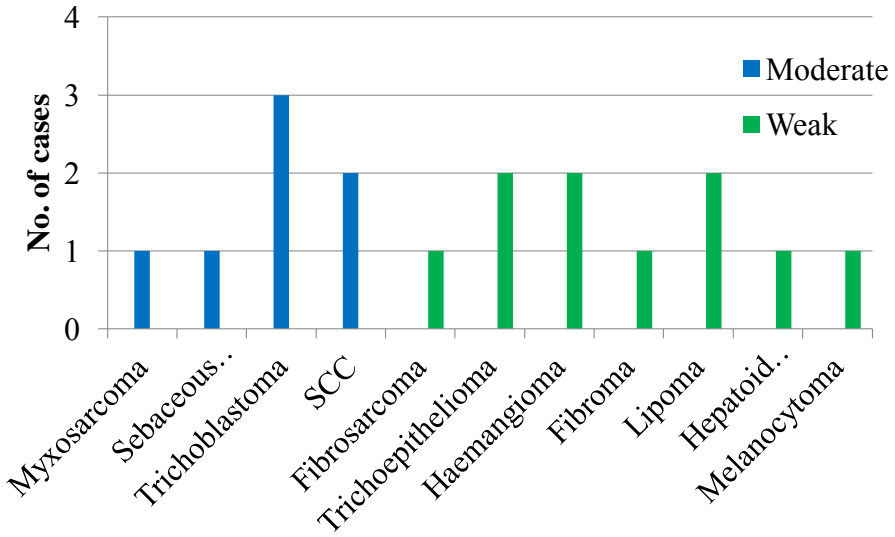

Fig. 25. Immunoexpression of DEPTOR in canine superficial tumours

4.8.4.1. Relationship between **DEPTOR** *expression and age of the animals*

Age-wise expression of DEPTOR in canine superficial tumours is given in Fig. 26. Statistical analysis revealed that there was no significant correlation between the age of the animals and DEPTOR expression in case of CSTs (r_s= -0.135, p= 0.606).

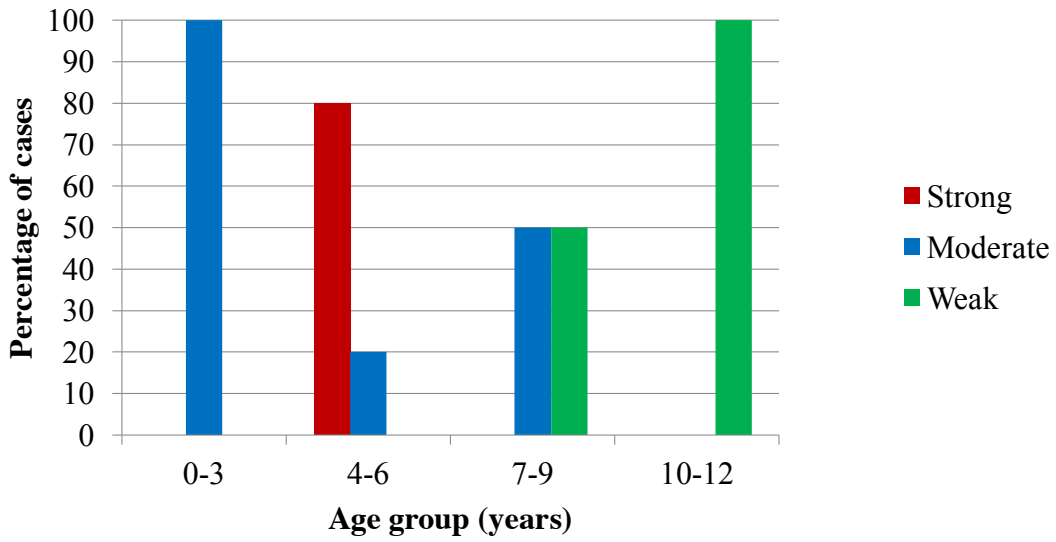

Fig. 26. Expression of DEPTOR in various age groups of dogs with superficial tumours

4.8.4.2. Relationship between **DEPTOR** *expression and size of superficial tumours*

Percentage of cases that showed strong, moderate and weak expression of DEPTOR based on the tumour size is represented in Fig. 27. There was poor correlation between the tumour size and DEPTOR expression in CSTs (r_s= 0.433, p= 0.082).

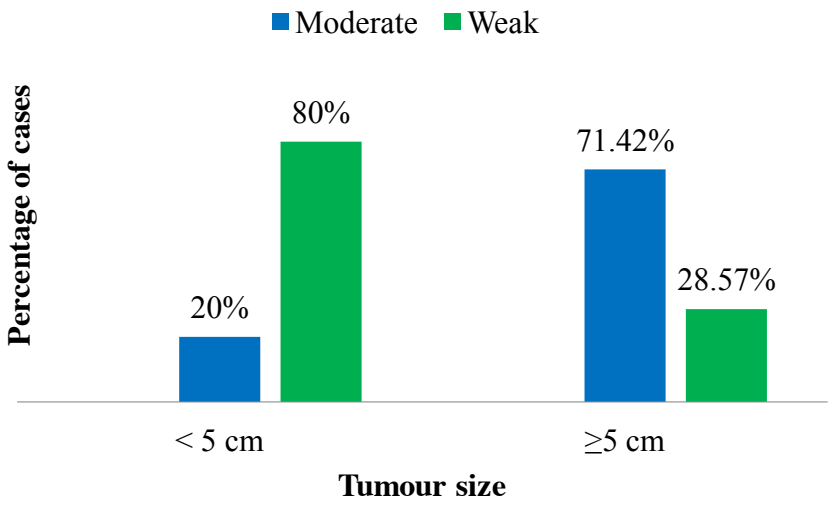

Fig. 27. Expression of DEPTOR according to the size of the tumours in canine superficial tumours

4.8.4.3. Relationship between expression of mTOR *and* DEPTOR *in superficial tumours*

Relative expression score of mTOR and DEPTOR in canine superficial tumours is shown in Fig. 28. It was observed that 35.30 per cent (6 cases) of the tumours showed strong expression for mTOR and moderate expression for DEPTOR (sebaceous adenoma, three cases of trichoblastoma and two cases of SCC). One case (fibrosarcoma) had strong mTOR and weak DEPTOR expression, another case (myxosarcoma) showed moderate expression for both. Four cases (two cases each of trichoepithelioma and haemangioma) had moderate expression for mTOR and weak expression for DEPTOR. Remaining five cases (fibroma, two cases of lipoma, hepatoid adenoma and melanocytoma) showed weak expression for both the proteins. The mean score of expression for mTOR in case of the canine superficial tumours (4.41±0.46) was significantly higher ($p<0.01$) than that of DEPTOR (3.24±0.36). Also there was strong positive correlation (Fig. 29) between expression of mTOR and DEPTOR in canine superficial tumours. ($r_s = 0.775$, $p<0.01$).

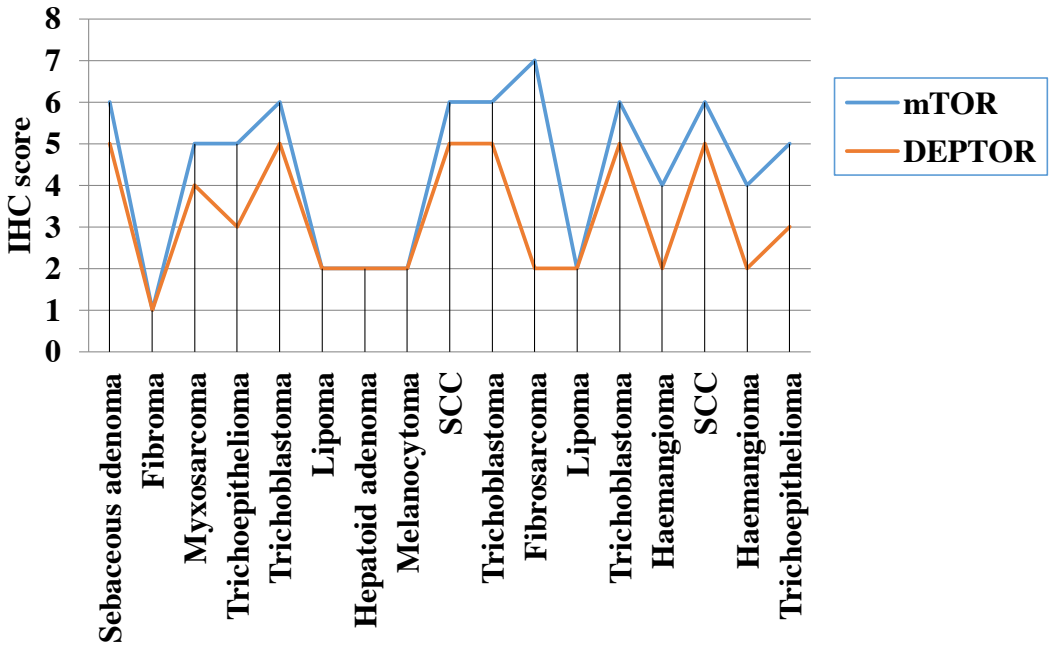

Fig. 28. Relative expression of mTOR and DEPTOR in canine superficial tumours

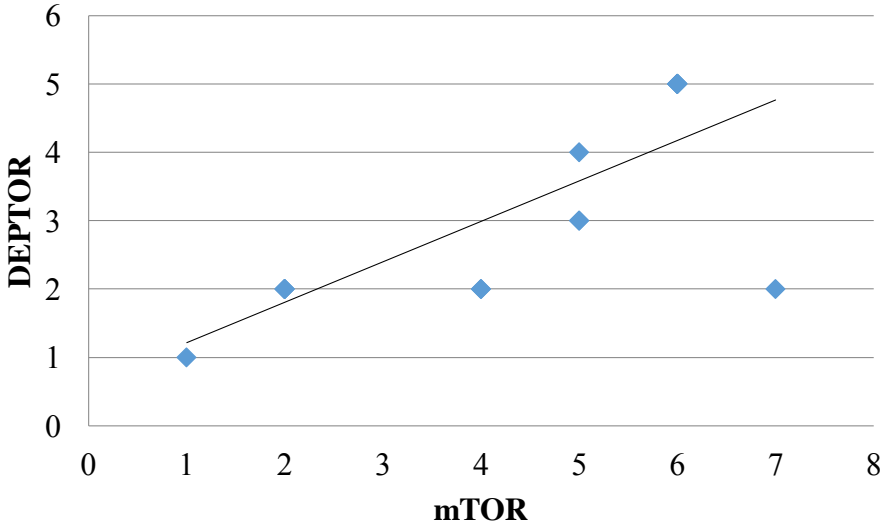

Fig. 29. Scatter plot showing positive correlation between expression of mTOR and DEPTOR in canine superficial tumours

5. DISCUSSION

One of the common diseases in dogs is cancer and it represents a major cause of mortality in canines. All breeds of dogs can be affected by tumour, although the etiology is multifactorial (Komazawa *et al*., 2016). Among all the tumours, the occurrence of skin and mammary tumours had been the highest (Reddy *et al*., 2009). Canine mammary tumour is considered as an excellent model to study the pathological and molecular characteristics of human breast cancer (Abdelmegeed and Mohammed, 2018). Mechanistic target of Rapamycin (mTOR) and Dishevelled EGL10 and Pleckstrin domain containing mTOR interacting protein (DEPTOR) are key proteins in the cell signaling pathway, which have been extensively studied in human context, but the data on their expression in canine tumours is meager. The current study was intended to assess the expression of these two proteins in canine mammary and superficial tumours and to elucidate their relative presence. The epidemiology of these tumours have also been studied, the data on which was beneficial to identify the breed-wise, sex-wise and age-wise risk of developing tumours and to assess the relationship between these epidemiological factors and the expression of mTOR and DEPTOR.

5.1. EPIDEMIOLOGY OF CANINE MAMMARY TUMOURS

In the present study, it was found that out of the 22 tumour suspected mammary gland excision biopsies, 81.81 per cent (n=18) were found to be malignant tumours. Benign and non-neoplastic lesions accounted for 18.18 per cent (n=4). This finding is in agreement with Dileepkumar *et al*. (2014) who recorded 83.33 per cent of CMTs as malignant and 16.67 per cent as benign. Salas *et al*. (2015) observed that out of the total suspected samples of CMTs, 47.8 per cent were benign tumours, 47.5 per cent were malignant tumours and 4.7 per cent were non-neoplastic lesions. This difference might be attributed to short period of study and small sample size.

5.1.1. Age-wise occurrence

In the present study, the highest occurrence of mammary tumour was observed in the seven to nine years age group (55 per cent). This is in agreement with the findings of Dileepkumar *et al*. (2014) who reported the maximum occurrence in the same age group. The mean age of the dogs affected with mammary tumours was 7.85±0.41 years.

It was found that age was a predisposing factor for development of mammary tumour which could be attributed to the hormonal changes associated with advancing age. Most of the tumours (80 per cent) were reported in dogs aged 7 years and above with the peak at 10 years. No case was reported in dogs below four years of age. This is in agreement with Bostock (1986) who reported that the occurrence of mammary tumours in dogs has shown a marked increase with advancing age, with the peak incidence at about 11 years, followed by a slight decrease thereafter.

5.1.2. Gender-wise occurrence

In the present study all the cases were reported in female dogs. This is in agreement with Moulton *et al*. (1970) and Gupta *et al*. (2012) who observed that mammary tumours were specific tumours of females and were rare in male dogs.

5.1.3. Breed-wise occurrence

In the present study the occurrence of CMTs was comparatively higher in Labrador (30 per cent) followed by German shepherd (25 per cent), Pomeranian (15 per cent), Non-descript (10 per cent) and Dachshund (10 per cent). Occurrence was low in case of Rottweiler and Doberman. Various authors have reported different breed-wise occurrence. Shivani (2007) observed highest occurrence in Pomeranian followed by German shepherd and Labrador retriever. Reddy *et al*. (2009) reported the highest occurrence in German Shepherd (25.0 per cent) followed by Spitz (24.22 per cent), Non-descript (19.53 per cent), Pomeranian (10.94 per cent), Labrador (6.25 per cent), Boxer (3.91 per cent), Doberman (4.69 per cent), Cocker Spaniel (3.13 per

cent), Bhutia (1.56 per cent) and Great Dane (0.78 per cent). Salas *et al*. (2015) observed the highest incidence of mammary tumours in poodles among the small breed category and German Shepherd among the large breeds. Thus, it could be concluded that breed predisposition to mammary tumours is not well established in the literature, which could be due to variation in sample size, popularity of particular breed in the geographical area and the difference in profile of the dogs at each study.

5.1.4. Tumour-wise occurrence

Among the mammary tumours, 90 per cent (18/20) were found to be malignant. The most frequent type of mammary tumour was ductal carcinoma (45 percent) followed by carcinoma arising in benign mixed tumour (CAMBT) (20 percent) and comedocarcinoma (10 per cent). Ten percent of the tumours were benign which were diagnosed as fibroadenoma. Solitary cases of spindle carcinoma, tubulopapillary carcinoma and solid carcinoma were also identified. Similar observations were made by Stratmann *et al*. (2008) who observed that the most common benign mammary tumour was adenoma and the most common malignant mammary tumour was carcinoma. Filho *et al*. (2010) also reported that the most frequent type of mammary tumour was simple carcinoma, both in necropsy and biopsy samples.

As per Horta *et al*. (2014) the most frequent malignant mammary neoplasm was carcinoma in mixed tumour (47.5 per cent) which differs from the observation of the present study. Even though variation in the subtype is of least significance since both these were carcinomas having similar prognosis and therapeutic management, further studies with larger sample size are required to find out the reason for increased incidence of any particular subtype.

5.2. GROSS PATHOLOGY OF CANINE MAMMARY TUMOURS

Grossly most of the tumours were observed as grayish to yellowish white in colour. Ductal carcinoma, solid carcinoma, comedocarcinoma and fibroadenoma

were soft to firm in consistency with varying shape. Kumar *et al*. (2011) reported that CMTs were usually elliptical to round in shape with consistency varied from soft to hard and cut surface showed grayish white colouration.

Carcinoma arising in benign mixed tumour was hard and difficult to be incised due to cartilaginous metaplasia and irregularity in shape. This is similar to the findings of Yogitha *et al*. (2015) who observed that mixed mammary tumours appeared lobular and consisted of bone or cartilage which imparted hard and rigid consistency to the tumour.

The size of the tumours recorded in the present study varied from one to 13 cm with the mean size 6.70±0.60 cm. As per Gabli *et al*. (2017), who evaluated 215 CMTs, the mean size of mammary tumour was 5.4±0.40 cm.

5.3. HISTOPATHOLOGY OF CANINE MAMMARY TUMOURS

Ductal carcinomas consisted of cords of pleomorphic neoplastic cells that surrounded slit like lumina which were lined by multiple layers of epithelial cells which exhibited significant cellular and nuclear pleomorphism. Fairly numerous mitotic figures were also observed. These are similar to the findings of Goldschmidt *et al*. (2011).

In carcinoma arising in benign mixed tumour (CABMT), focal areas of carcinomatous epithelial cells with marked cellular and nuclear pleomorphism were seen. Regions of cartilaginous metaplasia could also be noticed. According to Cassali *et al*. (2012), a mixed tumour consisted of both epithelial and mesenchymal parts, and was classified as CABMT when the epithelial component became carcinomatous and the mesenchymal component proliferates and undergoes metaplastic changes.

In comedocarcinomas, there were areas of necrosis in the centre of neoplastic cell aggregates. The neoplastic cells were arranged in sheets with fine fibrovascular stroma without any tubule differentiation. The necrotic centre was filled with eosinophilic material mixed with cell debris, neutrophils and macrophages. These findings were in agreement with Rasotto *et al*. (2012) and Mathew *et al*. (2019).

Spindle cell carcinoma was composed of islands of polygonal neoplastic cells with moderate pleomorphism supported by fine fibrovascular stroma. As reported by Alonso-Diez *et al*. (2019), spindle-shaped cells which resembled plump fibroblasts with elongated vesicular nuclei were numerous in the tumour. Proper lobular or glandular pattern was absent. As per Hong *et al*. (2014) some of the tumour cells in spindle carcinoma were arranged in nest pattern with atypical nuclei, a feature which was not evident in the present case.

Tubulopapillary carcinoma showed the tubular and papillary pattern of epithelial cell proliferation with delicate fibrovascular stalks as observed by Gamba *et al*. (2011). The cells showed moderate pleomorphism, and were arranged in multiple layers.

Solid carcinoma consisted of solid sheets of cells without lumina as described by Misdorp *et al*. (1972). The cells were oval, which had scant cytoplasm and poorly demarcated cell margins. Nuclei were hyperchromatic and oval with a single central basophilic nucleolus. Moderate anisocytosis and anisokaryosis with variable number of mitoses could be noticed.

In fibroadenoma the tubules were lined by columnar or cuboidal cells with a round and uniform nuclei. An extensive stroma of dense fibrous connective tissue was noticed. The fibroblastic cells had elongated nuclei, scant cytoplasm and inconspicuous cell margins. These features were in agreement with earlier report by Mathew *et al*. (2019).

Lobular hyperplasia was a condition characterized by non-neoplastic proliferation of intralobular ducts and acini. The epithelial cells showed atypical changes such as epithelial hyperplasia, nuclear hyperchromasia and moderate pleomorphism of the cells. Increased amount of inter lobular fibrous connective tissue was also seen. These findings were in accordance with Goldschmidt *et al*. (2011).

5.4. GRADING OF CANINE MAMMARY TUMOURS

All the malignant tumours were graded as per Clemente *et al*. (2010) to assess the malignancy of the tumour. The extent of tubule formation, nuclear pleomorphism and mitotic count were the factors considered to assign the grade to the tumours. The present study has shown that out of all the simple carcinomas, 85.71 per cent cases were graded as III or II whereas all the mixed tumours were classified as grade II. This observation was in agreement with Karayannopoulou *et al*. (2005) and Rezaie *et al*. (2009). The simple carcinomas are the most malignant type of mammary tumours which might be the reason for obtaining higher malignancy grading in most of them. It was evident that the histological malignancy grading was as effective tool in identifying the most malignant type of tumours and predicting the prognosis which is essential for prompt therapy.

5.5. EPIDEMIOLOGY OF CANINE SUPERFICIAL TUMOURS

The skin is the most common anatomical location of tumours in dogs and a wide range of tumour types could be found on the skin, subcutaneous tissue, and adenexa (Bronden *et al*., 2010). As per Pakrin *et al*. (2007) skin is an organ which is continuously exposed to several physical and chemical insults which make it prone to develop cancers.

5.5.1. Age-wise occurrence

In the present study it was found that majority (47.06 per cent) of the superficial tumours were reported in the age group of seven to nine years with the mean age of 6.55±0.59 years. This was in agreement with Babu *et al*. (2012) who observed highest incidence of tumours in dogs in the age group of five to eight years followed by nine to 12 years. Similar observations were made by Sharma *et al*. (2018), who observed the highest incidence of tumours in dogs in six to 10 year age group (60 per cent) followed by less than five years age group (24 per cent) and 10-

15 year age group (16 per cent). According to Chikweto *et al*. (2011) cutaneous neoplasms in dogs were encountered in relatively older dogs with the mean age of 7.6 years. The higher incidence of skin tumours in older animals might be attributed to the extended exposure of the skin to various environmental pollutants and sunlight. As per Goldschmidt and Goldschmidt (2017), the skin tumours are easily noticed by the owners, hence brought to the attention of the veterinarian, which could be a reason for increased occurrence of CSTs.

5.5.2. Gender-wise occurrence

It was seen in the present study that the occurrence of superficial tumours is comparatively higher in male dogs (58.82 per cent) than females (41.18 percent). This is in agreement with Dayananda *et al*. (2009) and Kashyap *et al*. (2013). Even though males had a higher incidence of skin tumours, no association between the sex and occurrence of superficial tumours could be observed in the current study which is consistent with the findings of Mukaratirwa *et al*. (2005), who observed that sex of dog had no effect on the likelihood of occurrence of skin tumours and chances of the tumour type to become malignant. According to Kaldrimidou *et al*. (2002) there was no significant sex predilection for skin tumours, but males were more affected by hepatoid gland adenoma, because of its association with androgen sex hormones.

5.5.3. Breed-wise occurrence

In this study the occurrence of superficial tumours was comparatively higher in Labradors (41.18 per cent) followed by Rottweiler (17.65 per cent), Non-descript (11.76 per cent), German shepherd (11.76 per cent) and Dachshund (11.76 per cent). One case was seen in a cross-bred dog. Sharma *et al*. (2018) also reported highest incidence (34 per cent) in Labrador. According to Babu *et al*. (2012) higher incidence of skin tumours was seen in German shepherd. As per Kaldrimidou *et al*. (2002) the chances of developing a skin tumour in a pure bred dog were two times higher than in mongrels and crossbreds.

Based on these observations, it could be inferred that purebred dogs has genetic predisposition to cancer development. Among the various subtypes of pure bred dogs, no specific breed predilection for skin tumours has been established which could be attributed to the variation in sample size and popularity of any particular breed in a geographical area.

5.5.4. Tumour-wise occurrence

The present study showed that majority (76.47 per cent) of the skin tumours was benign in nature. It is in accordance with the reports of Babu *et al*. (2012) and Kashyap *et al*. (2013). It was seen that 58.82 per cent of the tumours were of epithelial and melanocytic origin and 41.17 per cent were of mesenchymal origin. Sanja *et al* (2005) observed that epithelial and melanocytic tumours of the skin were the most common skin tumours in dogs. Simkus *et al*. (2016) found that out of the total neoplasms, 52.3 per cent were comprised of epithelial and melanocytic tumours and mesenchymal tumours constituted 47.7 per cent.

Among the epithelial and melanocytic tumours observed in the current study 50 per cent were hair follicle tumours. Variations existed in the prevalence of CSTs among the reports published from different parts of the world which might be a reflection of canine breed population (Simkus *et al*., 2016). Various environmental risk factors for development of CSTs were increased exposure to ultraviolet light due to year round warm humid climate and heavy load of ectoparasites and associated dermatitis in many dogs (Chikweto *et al*., 2011). From the present study it was not possible to draw a conclusion regarding the most prevalent canine skin tumour in this area. An exhaustive epidemiological study for long duration with large number of samples might be helpful to throw some light on the incidence pattern of these tumours and probable reasons.

5.6. GROSS AND HISTOPATHOLOGY OF CANINE SUPERFICIAL TUMOURS

The gross pathological features of the tumours varied with respect to the location, size, shape, colour and consistency. Some of the tumours showed specific features which were useful in identification of the tumour by gross examination.

Sebaceous adenoma was identified as an oval mass on the forelimb with firm consistency. The cut surface was solid with grayish white colour. Microscopic examination revealed sebaceous cells arranged in multiple lobules and separated by connective tissue trabeculae. These lobules had two types of cells, peripherally located undifferentiated generative (peripheral basaloid) cells and centrally located sebaceous cells showing varying degrees of sebaceous differentiation as observed by Ozyigit *et al*. (2005).

Fibroma was seen on the right hind limb as round subcutaneous mass with firm consistency and white colour on cut surface. These are in agreement with the findings of Abuseida *et al*. (2008). Microscopically it was composed of spindle-shaped and stellate fibroblasts. Nuclei of fibroblasts were oval, normochromatic and without mitotic figures. Abundant extracellular collagenous stroma was also found as reported by Behera *et al*. (2014).

Myxosarcoma appeared as multiple subcutaneous firm movable swellings on the left thigh. Surgical removal revealed four well encapsulated masses of irregular shape. Cut surface was soft and grayish white in colour with oozing of sticky fluid as observed by Hendrick (2017). The histopathological findings of myxosarcoma were similar to that observed by Headley *et al*. (2011). It was characterised by proliferation of spindle shaped fibroblasts bathed in an abundant myxoid matrix which stained blue. The neoplastic cells had indistinct cellular margins and marked pleomorphism of cells and nuclei. The high nuclear density was the reliable feature for differentiating it from myxoma because the variation in other features among the tumours is subtle.

Two cases of trichoepithelioma were studied. Both the growths were observed on the dorsum which were ulcerated and well demarcated. The masses were four to five cm in size, with soft consistency and grey colour. The cut section showed grayish white granular tissue. As per Beck *et al*. (2016) the most common site of trichoepithelioma was the back and the average diameter was 3.75 cm. They have also observed that the tumours were presented as nodules or warts with alopecia and ulceration. Histologically trichoepitheliomas were composed of cysts filled with keratinous debris. The basal lamina was thickened and eosinophilic, with palisaded cells having little cytoplasm and hyperchromatic nuclei. Towards the centre of the cyst, extensively keratinized cells were observed. These findings were similar to that reported by Raval *et al*. (2015).

Three cases of trichoblastoma were examined during the study. The size varied from five to eight cm. Two masses were observed on the forehead and one on the neck. The masses were well encapsulated, round to oval in shape with firm consistency. The cut surface showed grayish white colour. These findings are in accordance with Sawale *et al*. (2015). Microscopically the trichoblastomas were multilobulated with ribbon type cords of branching and anastomosing cells. The cells showed a palisaded appearance with little cytoplasm and prominent nuclei which appeared hyperchromatic, with inconspicuous nucleoli. The scant cytoplasm was eosinophilic and cell borders were not prominent. Scattered atypical mitotic figures were seen in moderate numbers. Moderate quantity of stroma found between the cords of cells. These findings were in consonance with Campos *et al*. (2014).

Lipomas were non-encapsulated soft round masses. The cut surface had white colour and greasy texture resembling fat as reported by Simeonov *et al*. (2011). Microscopically it was characterised by proliferating adipocytes that had single, clear and large cytoplasmic vacuole with the nuclei compressed to the periphery as described by Leriquier *et al*. (2017). Few infiltrating neutrophils could be seen with no evidence of necrosis.

Hepatoid gland adenoma was a solitary pedunculated mass in the perianal region. The cut surface was soft and grayish white in colour with focal hemorrhages. Histologically it was characterised by cords of cells with similarity to normal hepatocytes. The cells had abundant eosinophilic cytoplasm with centrally placed, large vesicular and normochromatic nuclei. Basaloid reserve cells with scant cytoplasm and hyperchromatic nuclei, at the periphery of the lobules were characteristic. The morphological and microscopical findings were in agreement with Goldschmidt and Goldschmidt (2017) who observed that most of hepatoid gland neoplasms (88 per cent) were seen in the perianal region with size varying from 0.5 to five cm.

Melanocytoma was observed as a round ulcerated mass in the interdigital space of the left forelimb with firm consistency and black colour. These findings were in agreement with Spangler and Kass (2006) who reported cutaneous melanoma in dogs between toes. Goldschmidt and Goldschmidt (2017) also observed that melanocytomas varied in size from small macules to large masses with almost 5 cm diameter and the colour varied from black through shades of brown or grey depending on the quantity of melanin pigment within the neoplastic cells. Microscopically it was characterised by presence of abundant quantity of melanin pigment in the cytoplasm of neoplastic melanocytes, which obscured the nuclear morphology. These characteristics are similar to that observed by Abuseida *et al*. (2008)

Two cases of squamous cell carcinoma were studied. One case was on the eye lid and the second on digit of the left forelimb. The tumours were ulcerated, firm to hard in consistency with reddish brown colour. These findings were in conformity with Chandrashekaraiah *et al*. (2011). As per Henry *et al*. (2005) the most common tumour type diagnosed among the digital tumours in dogs was squamous cell carcinoma. Microscopically, it was observed that both the cases differed in the degree of differentiation. One was a case of well differentiated squamous cell carcinoma

characterised by nests and cords of neoplastic squamous cells with extensive keratosis and formation of distinct keratin “pearls”. In the less differentiated squamous cell carcinoma, the squamous epithelial cells were smaller in size and the keratin formation was indistinguishable. These findings were in conformity with the reports of Chandrashekaraiah *et al*. (2011) and Belluco *et al*. (2013)

The case of fibrosarcoma appeared as round hard and solid nodular mass which was protruding out of the vagina. It was hard to cut with creamy white colour on the cut surface. These findings were in similar to the observations made by Al-Kenanny *et al*. (2013) and Mumba *et al*. (2013). Histologically it was characterized by spindle shaped tumour cells and collagen fibers arranged in herringbone pattern characterized by architectural disarray. These features are in agreement with the reports of Mukhopadhayay *et al*. (2012) and Al-Kenanny *et al*. (2013). Cytoplasm was scant and nuclei were elongated to oval with inconspicuous nucleoli. High nuclear density and presence of less collagen differentiated it from fibroma as described by Hendrick (2017).

Haemangiomas were observed on the abdomen and hind limb with an average size of three cm. The growths were red in colour with soft to firm consistency. The cut surface also was red in colour with blood oozing out. Similar observations were made by Balachandran *et al*. (2014) and Sawale *et al*. (2014). Microscopically they were consisted of widely dilated and variable sized vascular spaces filled with erythrocytes. These were in accordance with the report of Balachandran *et al*. (2014). Organised thrombi with foci of haemosiderosis were found in the tumour. A clear fibrous connective tissue stroma, infiltrated by inflammatory cells, separating the vascular channels was seen. These findings are in accordance with Sasani *et al*. (2015).

5.7. IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed to study the expression of mTOR and DEPTOR in canine mammary and superficial tumours. Semiquantitative method based on the intensity and percentage of cells showing positivity was used for scoring of immunohistochemical staining as described by Vakkala *et al*. (1999).

5.7.1. Mechanistic target of Rapamycin (mTOR) expression in CMTs

In the present study, immunohistochemical expression of mTOR was evaluated in 20 CMTs. It was seen that, all the tumours had higher expression of mTOR when compared to the normal mammary gland ($p<0.01$), although the staining intensity varied. Eighty five per cent of the tumours had strong to moderate positivity whereas 15 per cent showed weak staining. All the malignant tumours showed moderate to strong expression of mTOR except a case of ductal carcinoma that had weak expression. The fibroadenoma cases showed weak immunostaining against mTOR.

Although the reports on mTOR expression in CMTs are meager, Delgado *et al*. (2015) evaluated the presence of phospho-mTOR, the activated form of mTOR, in 45 canine mammary carcinomas and observed that normal mammary gland showed absence of p-mTOR, whereas 78 per cent of the mammary carcinomas exhibited immunoreactivity for this protein. They concluded that p-mTOR expression is directly related to the neoplastic transformation of mammary gland. Maniscalco *et al*. (2013) demonstrated that the feline triple negative mammary carcinomas showed high expression of mTOR.

5.7.1.1. Relationship between age group of the animals and **mTOR** *expression*

The age of the animals reported with mammary tumours ranged between four and 10 years with the mean age of 7.85±0.41 years. It was observed that there was no correlation between the age of the animals and mTOR expression. Ueng *et al*. (2012) observed that the expression of p-mTOR is not correlated with the age of the patients

in triple negative breast carcinomas in humans. However Bajwa *et al*. (2017) observed that the age associated pathological changes in the female reproductive tract in humans could be attributed to aberrant mTOR signaling. Aged animals were more prone to development of mammary tumours; the reason could be multifactorial (Goldscmidt *et al*., 2017). In the present study mTOR was found to be expressed in all mammary tumours irrespective of the age of the animals. However further studies with larger sample size might be required to delineate the influence of age on mTOR expression.

5.7.1.2. Relationship between tumour grade and **mTOR** *expression*

The present study showed that there was poor correlation between mTOR expression and the grade of the tumours. This was in agreement with previous reports by Maniscalco *et al*. (2013) wherein no significant correlation was observed between tumour grade and mTOR expression in triple negative feline mammary carcinomas. Delgado *et al*. (2015) also reported no significant correlation between tumour grade and p-mTOR expression in CMTs.

5.7.1.3. Relationship between the tumour size and **mTOR** *expression in mammary tumours*

In the present study there was no significant correlation between the size of the tumours and expression of mTOR. Maniscalco *et al*. (2013) did not report any significant correlation between tumour size and mTOR expression in feline triple negative mammary carcinomas. Cao *et al*. (2016) did not observe any correlation between the tumour size and over expression of mTOR in human gastric carcinomas.

5.7.2. Expression of DEPTOR in canine mammary tumours

The present study revealed that DEPTOR was expressed significantly in mammary tumours when compared to the normal mammary gland ($p<0.01$). Moderate to weak expression was observed in all the tumours with the exception of a case of CABMT wherein strong immunostaining was observed in the epithelial

component. Wang *et al*. (2012) reviewed that in human cancers DEPTOR played a dual role of either tumour suppressor or tumour promoter, depending on the type tumour.

5.7.2.1. Relationship between age group of the animals and **DEPTOR** *expression*

In the present study no significant correlation was observed between the age group of the animals affected with mammary tumour and the intensity of DEPTOR expression. Age factor did not appear to be correlated with the expression of DEPTOR in colorectal cancer (Lai *et al*., 2014) and esophageal carcinoma (Dong *et al*., 2017) in humans.

5.7.2.2. Relationship between tumour grade or size and **DEPTOR** *expression*

There was poor correlation between the expression of DEPTOR and tumour grade or size. In a meta-analysis study, on the expression of DEPTOR in various human cancers including breast cancer Hu *et al*. (2018) concluded that the various clinicopathological factors including tumour grade and tumour size had no correlation with DEPTOR expression.

5.7.3. Relationship between mTOR and DEPTOR expression in CMTs

On comparison of the immunoscore for mTOR and DEPTOR expression in CMTs, it was evident that the mean expression of mTOR in CMTs was significantly higher than the mean expression of DEPTOR ($p<0.01$). It was also found that there was significant positive correlation between the expression of mTOR and DEPTOR in CMTs. Peterson *et al*. (2009) observed that DEPTOR specifically interacts with mTOR and functions as an endogenous inhibitor of mTOR. Catena *et al*. (2017) reviewed the role of DEPTOR in human cancers. The authors reported that DEPTOR had a tumour suppressor role in pancreatic ductal adenocarcinoma, lung adenocarcinoma, liver cancer, colorectal cancer and triple-negative breast cancers whereas it functioned as an oncogene in some other tumour types such as differentiated thyroid carcinoma, multiple myeloma, cervical squamous cell

carcinoma and certain triple-negative breast cancers. The dual role of DEPTOR as an oncosuppressor and oncogene in human breast cancers was reported by Parvani *et al*. (2015).

In the present study the mean expression of mTOR was significantly higher than DEPTOR and there was a significant positive correlation between the expressions of these two proteins. Thus it could be assumed that DEPTOR had potent oncogene role in canine mammary tumours. Further studies employing larger sample size would be required to elucidate the expression pattern and mechanism in individual tumour types.

5.7.4. Mechanistic target of Rapamycin (mTOR) expression in canine superficial tumours

In the present study it was seen that mTOR was strongly expressed in sebaceous adenoma, trichoblastoma, squamous cell carcinoma and fibrosarcoma and moderately expressed in myxosarcoma, trichoepithelioma and haemangioma. However, in other tumours which included fibroma, lipoma, hepatoid adenoma and melanocytoma, mTOR was weakly expressed.

Sardina *et al*. (2019), after studying the hair follicle tumours in humans, observed that mTOR activation had a crucial role in the follicle tumourigenesis. In the present study also, all the trichoblastomas have shown strong expression for mTOR and the trichoepitheliomas showed moderate expression.

The squamous cell carcinomas examined in the present study have shown strong mTOR expression. Mastronikolis *et al*. (2017) observed that mTOR is over activated in the neoplastic cells of oral squamous cell carcinoma in humans. Li *et al*. (2016) studied the expression of mTOR and p-mTOR in human oesophageal squamous cell carcinoma and observed that the expression of mTOR and p-mTOR correlated with unfavourable outcomes on TNM stage, tumour invasion and degree of differentiation.

The expression of mTOR was weak in fibroma case. On the contrary Musha *et al*. (2018) observed that the odontogenic fibroma in a human patient was moderately positive for mTOR.

Murai *et al*. (2012) observed that mTOR pathway is activated in both canine haemangiomas and haemangiosarcomas with higher mean expression in the latter cases. Present study also demonstrated that the two reported cases of haemangioma had moderate expression of mTOR.

Present study evaluated the expression of mTOR in a case of melanocytoma and found that mTOR was not expressed in this case. This contrasts with the study of Kent *et al*. (2009) wherein mTOR was significantly expressed in canine malignant melanoma cell lines.

5.7.4.1. Correlation between **mTOR** *expression and clinicopathological parameters such as age of the animals and tumour size*

The present study has revealed that there was no significant correlation between the age of the animals and size of the tumours with mTOR expression in canine superficial tumours.

5.7.5. Expression of DEPTOR in superficial tumours

Analysis of DEPTOR expression in CSTs has revealed that none of the cases were strongly positive. However sebaceous adenoma, trichoblastoma, squamous cell carcinoma and myxosarcoma have shown moderate expression. Other tumours such as trichoepithelioma, haemangioma, fibroma, lipoma, hepatoid adenoma, melanocytoma and fibrosarcoma were weak in DEPTOR immunoexpression. Liu *et al*. (2015) observed significant increase in DEPTOR expression in human esophageal squamous cell carcinoma.

5.7.5.1. Correlation between **DEPTOR** *expression and clinicopathological parameters such as age of the animals and tumour size*

In the present study it was observed that there was no significant correlation between tumour size and age group of the animals with the expression of DEPTOR. Ji *et al*. (2016) observed that the expression of DEPTOR in human esophageal carcinoma was related to neither the age of the patients nor the tumour size.

5.7.6. Relationship between mTOR and DEPTOR expression in canine superficial tumours

The mean expression of mTOR was significantly ($p<0.01$) higher than the mean expression of DEPTOR in canine superficial tumours. It was also seen that there was a significant positive correlation between mTOR and DEPTOR expression in these tumours. Thus it could be concluded that DEPTOR expression is increased in canine superficial tumours along with mTOR pointing towards its potent oncogene role. Similar studies in human medicine had revealed oncogene role of DEPTOR in esophageal squamous cell carcinoma (Liu *et al*. 2015), hepatocellular carcinoma (Yen *et al*., 2012) and osteosarcomas (Hu *et al*., 2018).

The present study attempted to evaluate the expression of mTOR and DEPTOR in canine mammary and superficial tumours and to delineate the relationship between their expressions. The relationship between various clinico-pathological factors like age of the animals, tumour grade and tumour size and expression of these proteins have also been studied. Similar results were reported with mTOR and DEPTOR expression in various tumours of human beings. However studies on the relative expression of these two critical proteins in the cell signaling pathway were absent in veterinary medicine and to our knowledge, this is the first attempt to study this in veterinary medicine. Further studies with larger sample size are required to ascertain the role of these proteins in canine cancer biology and to use them as potential prognostic markers and therapeutic targets in various tumours.

6. SUMMARY

The present work was undertaken to study the expression of two proteins, critical in the cell signaling pathway namely, mechanistic target of Rapamycin (mTOR) and Dishevelled, EGL10 and Pleckstrin domain containing mTOR interacting protein (DEPTOR) in canine mammary and superficial tumours. Along with that, the epidemiology, gross pathology and histopathology of the tumours have also been studied.

Excision biopsy samples collected from University Veterinary Hospitals, Mannuthy and Kokkalai during the period from January to December 2018 were utilised for the study. The tissues were collected in 10 per cent NBF for histopathology and immunohistochemistry. Detailed epidemiological data including the age, sex, breed of dogs and various gross features of the tumours like size, shape, location, colour and consistency were recorded.

Twenty canine mammary tumours and 17 canine superficial tumours were utilized for the study. Age wise analysis revealed that dogs affected with mammary tumours and superficial tumours had a mean age of 7.85±0.41 years and 6.55±0.59 years respectively. The highest occurrence of both the tumours was seen in the age group of 7 to 9 years. All the mammary tumours were reported in female dogs but in case of superficial tumours male dogs (58.82 per cent) were more affected. Data on breed wise tumour occurrence showed the highest occurrence of both the type of tumours in Labradors.

Among the CMTs, 90 per cent were malignant, which comprised of ductal carcinoma, comedocarcinoma, carcinoma arising in benign mixed tumour, tubulopapillary carcinoma and solid carcinoma; 10 per cent were benign, which were diagnosed as fibroadenoma. In case of superficial tumours majority (76.47 per cent) were benign in nature. They were identified as trichoblastoma, lipoma, sebaceous adenoma, fibroma, trichoepithelioma, hepatoid adenoma, melanocytoma, and

haemangioma. The remaining malignant tumours were comprised of myxosarcoma, squamous cell carcinoma and fibrosarcoma.

Grossly, CMTs varied in shape, colour and consistency with a mean size of 6.70±0.60cm (range one to 13 cm). The superficial tumours had a mean size of 4.65±0.37cm (range three cm to eight cm). They also varied in shape, colour and consistency, although, predominantly they had round to oval shape with grayish white colour. The gross pathological features could be of some help to identify the tumour types like melanoma, lipoma and carcinoma arising in benign mixed tumour, but they could not be completely relied on. Histopathology, which is considered as the gold standard for tumour diagnosis, has been extensively employed in the current study for identification of the tumour types.

On histological malignancy grading of the mammary tumours, it was seen that out of the total 18 malignant mammary tumours, four were classified as grade III (high grade or poorly differentiated), eight were classified as grade II (medium grade or moderately differentiated) and the remaining six were classified as grade I (low grade or well differentiated). Majority of the simple carcinomas, which are the most malignant type, had higher grade of III or II.

Immunohistochemistry for mTOR and DEPTOR revealed that both the proteins were significantly expressed ($p<0.01$) in both mammary and superficial tumours. The age of the animals was found to have no correlation with the expression of mTOR and DEPTOR in both these type of tumours. Clinicopathological factors like tumour size and tumour grade also had no relation with the expression of the proteins.

Statistical analysis revealed that the mean expression of mTOR in mammary tumours and superficial tumours was significantly higher than the corresponding mean expression of DEPTOR in these tumours. Further, it was also found that there was a significant positive correlation between the expressions of mTOR and DEPTOR in both these type of tumours.

Thus, the present study identified that the two key proteins in cell signaling pathway, the mTOR and DEPTOR, were significantly expressed in canine mammary and superficial tumours. Further it was shown that their expression was positively correlated. Hence the oncogene role of DEPTOR was established through the current study. This study might pave the way for identifying these proteins as potent therapeutic targets in canine tumours. However further studies with larger sample size are warranted to ascertain the role of these proteins in canine cancer biology and to use them as potential prognostic markers and therapeutic targets in various tumours.

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EXPRESSION OF MECHANISTIC TARGET OF RAPAMYCIN (mTOR) AND DISHEVELLED, EGL 10 AND PLECKSTRIN DOMAIN CONTAINING MTOR INTERACTING PROTEIN (DEPTOR) IN CANINE SUPERFICIAL AND MAMMARY TUMOURS

RAIMON MATHEW

(17-MVM-52)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

(Veterinary Pathology)

2019

Faculty of Veterinary and Animal Sciences

Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY PATHOLOGY

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR, KERALA, INDIA, 680651

ABSTRACT

The present work was undertaken to study the expression of two proteins, critical in the cell signaling pathway namely, mechanistic target of Rapamycin (mTOR) and Dishevelled, EGL10 and Pleckstrin domain containing mTOR interacting protein (DEPTOR) in canine mammary and superficial tumours. Excision biopsy samples of mammary and skin tumour suspected growths from dogs presented to University Veterinary hospitals, Mannuthy and Kokkalai were collected for the study during the period from January 2018 to March 2019. Out of the 129 tumour suspected cases, 22 were mammary tumour growths and 17 were superficial tumour growths. Age wise analysis revealed that the mean age of the dogs affected with mammary tumours and superficial tumours was 7.85±0.41 years and 6.55±0.59 years respectively. The highest incidence of both the tumours was seen in the age group of seven to nine years. All the mammary tumours were recorded in female dogs but in case of superficial tumours male dogs (58.82 per cent) were more affected. Data on breed-wise occurrence showed the highest occurence of both the type of tumours in Labradors. Among the mammary tumours, 90 per cent were malignant and 10 per cent were benign, where as in case of superficial tumours majority (76.47 per cent) were benign in nature. Grossly CMTs varied in shape, colour and consistency with a mean size of 6.70±0.60 cm. The superficial tumours were predominantly round to oval in shape with grayish white colour having a mean size of 4.65±0.37cm. On grading of malignant CMTs, majority of the simple carcinomas, which are the most malignant type, had higher grade of II or III. Immunohistochemistry for mTOR and DEPTOR has revealed that both the proteins were significantly expressed ($p<0.01$) in both CMTs and CSTs. Clinico-pathological factors like age of the animals, tumour size and tumour grade had no relation ($p>0.05$) with expression of these proteins. The mean expression of mTOR in mammary tumours and superficial tumours was significantly higher ($p<0.01$) than that of DEPTOR. Further, it was also found that

there was significant positive correlation ($p<0.01$) between the expression of mTOR and DEPTOR in both these type of tumours. Thus, the present study identified that the two key proteins in cell signaling pathway, the mTOR and DEPTOR, were significantly expressed in canine mammary and superficial tumours. Further it was shown that their expression was positively correlated. Hence the oncogene role of DEPTOR in CMTs and CSTs was established through the study.

Acad. Form No. 19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

Faculty of Veterinary and Animal Sciences

PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS DEGREE

1. Title of thesis:

Expression of mechanistic target of Rapamycin (mTOR) and Dishevelled, EGL10 and Pleckstrin domain containing mTOR interacting protein (DEPTOR) in canine superficial and mammary tumours

(a) Title of the departmental/ KVASU research project of which this forms a part:

Not applicable

(b) Code No. if any, and order by which the departmental/KVASU research project is approved:

Not applicable

2. (a) Name of student:

Raimon Mathew

(b) Admission No:

17-MVM-52

(c) Name of the Discipline:

Veterinary Pathology

3. (a) Name of Major Advisor (Guide)

Dr. Sajitha I.S.

(b) Designation:

Assistant Professor,

Department of Veterinary Pathology,

College of Veterinary and Animal Sciences,

Mannuthy, Thrissur, 680 651

5. Objectives of the study:

1. Expression of mTOR and DEPTOR proteins by immunohistochemistry(IHC) in canine superficial and mammary tumours

2. Relative presence of mTOR and DEPTOR proteins in canine superficial and mammary tumours

6. Practical/ Scientific utility:

Research into signalling pathways involved in cancer progression has led to many discoveries of which mechanistic target of rapamycin (mTOR) is a key player. While mTOR is necessary for normal physiology, cancer cells take advantage of mTOR signaling to drive their metabolic transformation, neoplastic growth and increased metastatic potential. It has been reported that, DEPTOR is an important protein which can inhibit mTOR. In many human tumors including breast cancer, mTOR is hyperactivated. However, DEPTOR has been reported to be down regulated in some types of human cancers and over expressed in some other tumor

types including breast cancer. No reports are available so far on the expression and the interaction of mTOR and DEPTOR proteins in canine superficial and mammary tumours.

Hence, the present study is designed to assess the association between mTOR and DEPTOR protein expression in canine superficial and mammary tumours. The results can be utilised further for combined anticancer therapy in dogs targeting mTOR signalling pathways.

7. Important publications on which the study is based:

Rubio-Viqueira and Hidalgo (2006) reported that mTOR inhibitors are promising anticancer agents.

Guertin and Sabatini (2007) concluded that the mammalian target of rapamycin (mTOR) has emerged as a critical effector in cell-signalling pathways commonly deregulated in human cancers.

Peterson *et al*. (2009) reported that DEPTOR over expression in multiple myeloma is necessary for activating PI3K/Akt signalling and promoting cell survival.

Pinho *et al*. (2012) reported that canine tumours are spontaneous animal models of human carcinogenesis.

Wang *et al*. (2012) stated that DEPTOR has been found to be over expressed in many tumour types including breast cancer.

Maniscalco *et al*. (2013) demonstrated high expression of mammalian target of rapamycin(mTOR) in triple negative feline mammary carcinomas.

Lai *et al*. (2014) reported that DEPTOR expression negatively correlates with mTORC1 and tumour progression in colorectal cancer.

Delgado *et al*. (2015) found that p-mTOR is widely expressed in mammary carcinomas and play a pivotal role in mammary carcinogenesis in bitches.

Rad *et al*. (2018) concluded that mTOR is a master regulator of cell growth control and is often activated in cancer.

8. Outline of the technical programme:

A minimum of 24 canine superficial and mammary tumour necropsy and excisional biopsy samples will be collected from the cases presented to Department of Veterinary Pathology, CVAS, Mannuthy, University Veterinary hospitals, Mannuthy and Kokkalai and nearby Government Veterinary hospitals. Clinical history of the affected dog will be collected and gross examination of the tumours will be done. Visceral organs with or without macro metastasis will also be collected during postmortem examination. All the samples will be collected in 10 percent neutral buffered formalin and processed for histopathological examination as per Bancroft

and Gamble (2008). The tumours will be classified and graded based on gross and histopathology as per Cullen e*t al*. (2002).

The presence of mTOR and DEPTOR proteins in tumour tissues will be assessed by immunohistochemistry (IHC) using commercially available kits. Based on the percentage of positive cells, the immunolabelled sections will be scored as per Vakkala *et al*. (1999) and relative presence of mTOR and DEPTOR will be studied. Data will be analysed using SPSS version 24.

9. Main items of observations to be made:

1. Gross and histopathological changes in primary tumours and visceral organs
2. Types and grades of the tumours
3. Expression of mTOR and DEPTOR proteins in tumour tissues by IHC

10. Facilities

(a) Existing

With the existing facilities in the Department of Veterinary Pathology and Central Instruments Laboratory of the college

(b) Additional facilities required

Chemicals and biologicals

11. Duration of study

Four semesters

12. Financial estimate

Biologicals, miscellaneous and contingencies	Rs. 25,000
Total	Rs. 25,000

Signature of student

Signature of Major Advisor

Place: Mannuthy

Date: 25.06.18

Name, Designation and signature of Members of Advisory Committee

Chairperson

Dr. Sajitha I.S.
Assistant Professor,
Department of Veterinary Pathology,
College of Veterinary and Animal Sciences,
Mannuthy, Thrissur, 680 651

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APPENDIX-1

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APPENDIX II

Time frame of work

Semester I

1. Planning of the programme for research
2. Collection of literature
3. Preparation of synopsis

Semester II

1. Collection of samples
2. Commencement of research work

Semester III

1. Collection of samples
2. Continuation of research work

Semester IV

1. Completion of research work
2. Analysis of results
3. Compilation, preparation and submission of thesis

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to Animals Act (Amendment, 1998)

Place: Mannuthy
Date : 25.06.18

Dr. Sajitha I.S.
Major Advisor

Curriculum Vitae

1. Name of candidate : Dr. (Maj.) Raimon Mathew
2. Date of birth : 21-10-1981
3. Place of birth : Kottayam
4. Marital status : Married
5. Permanent address : Nathania, Maveli Nagar
   Kanakkary P.O. 686632
6. Major field of specialization : Veterinary Pathology
7. Educational status : BVSc &AH, undergoing MVSc
8. Professional experience : Serving Officer of Remount and Veterinary Corps of Indian Army
9. Publications made : Mathew, R., Sajitha, I.S., Nair, S.S., Krishna, D. and Abraham, M.J. 2019. Canine mammary tumours: Histological malignancy grading as a prognostic indicator. *Pharma Innovation*. **8**: 149-151.
10. Membership of Professional societies:
    a) Member, Kerala State Veterinary Council
    b) Member, Equestrian Federation of India
    c) Member, Indian Association of Veterinary Pathologists

Place: Mannuthy

Date:

Plate 1. Mammary tumour affecting the right caudal thoracic and left caudal abdominal mammary glands

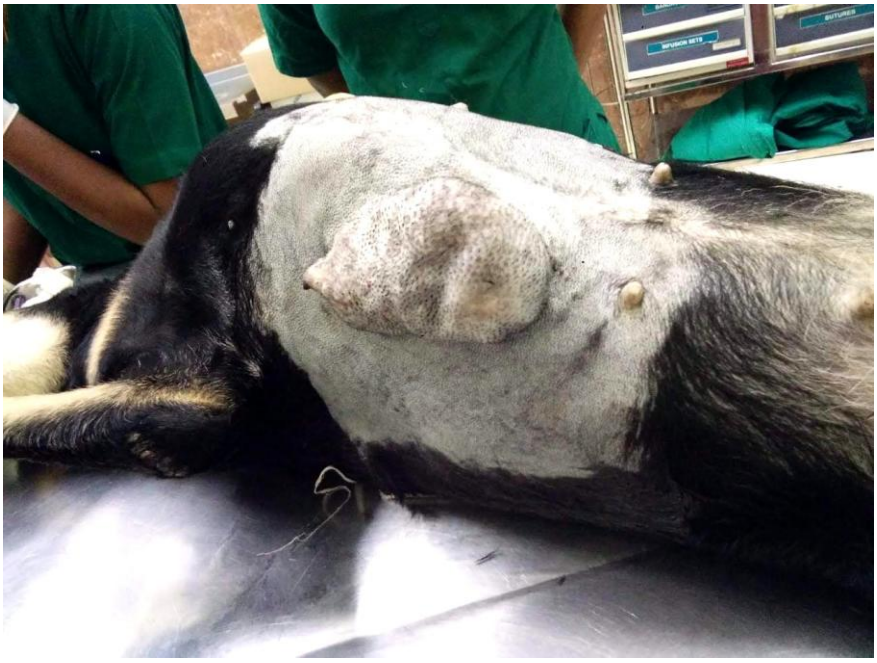

Plate 2. Mammary tumour in the right caudal thoracic gland

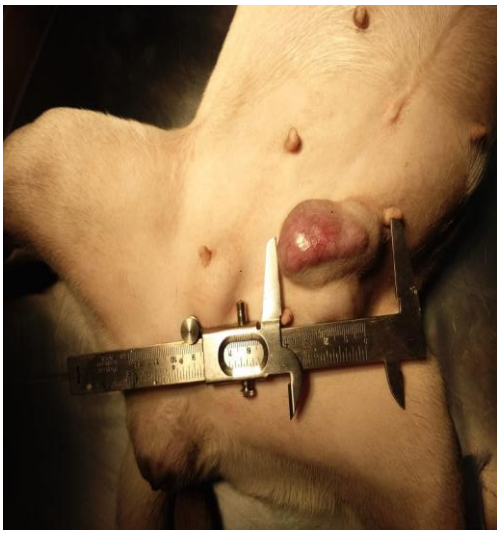

Plate 3.A. Mammary tumour in the left caudal abdominal gland

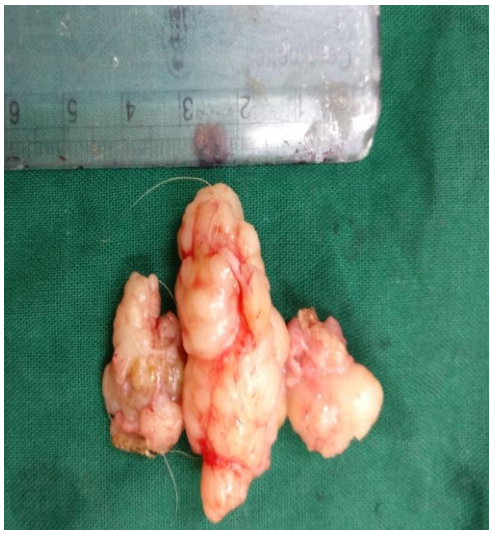

Plate 3.B. Irregular shaped tumour masses

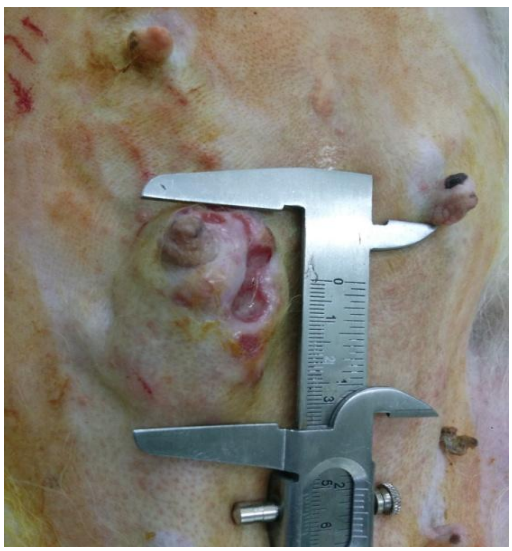

Plate 4.A. Mammary tumour in the left caudal thoracic gland

Plate 4.B. Mammary tumour. Cut surface with grayish white colour

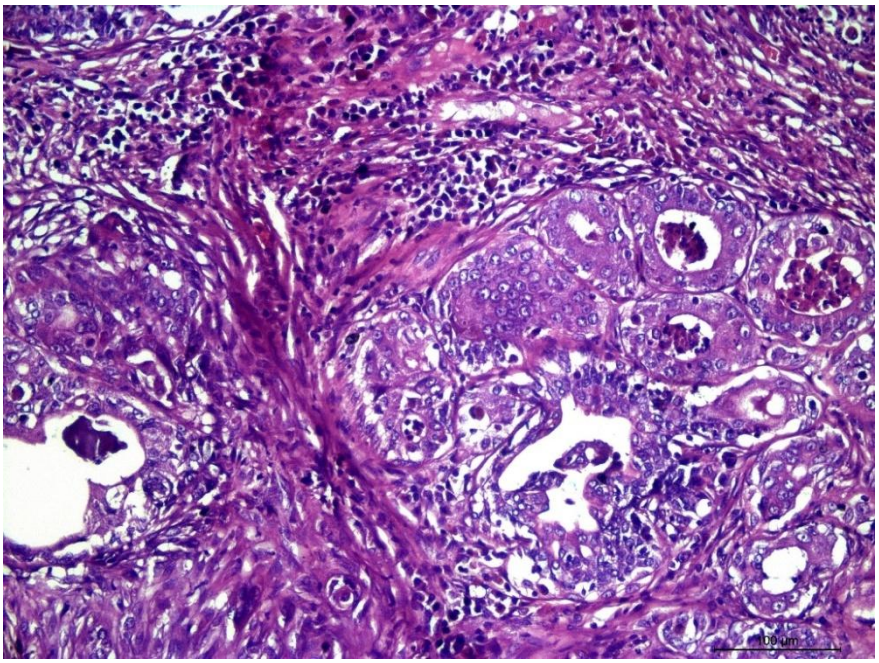

Plate 5. Ductal carcinoma. Pleomorphic neoplastic epithelial cells proliferating in multiple layers (H&E x200)

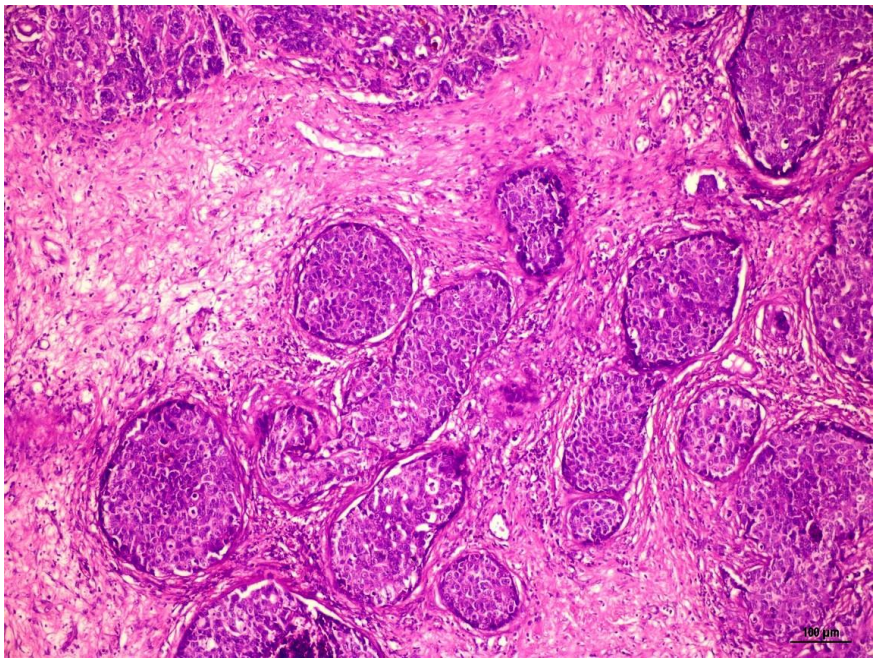

Plate 6. Solid carcinoma. Solid sheets of neoplastic cells without lumina (H&E x100)

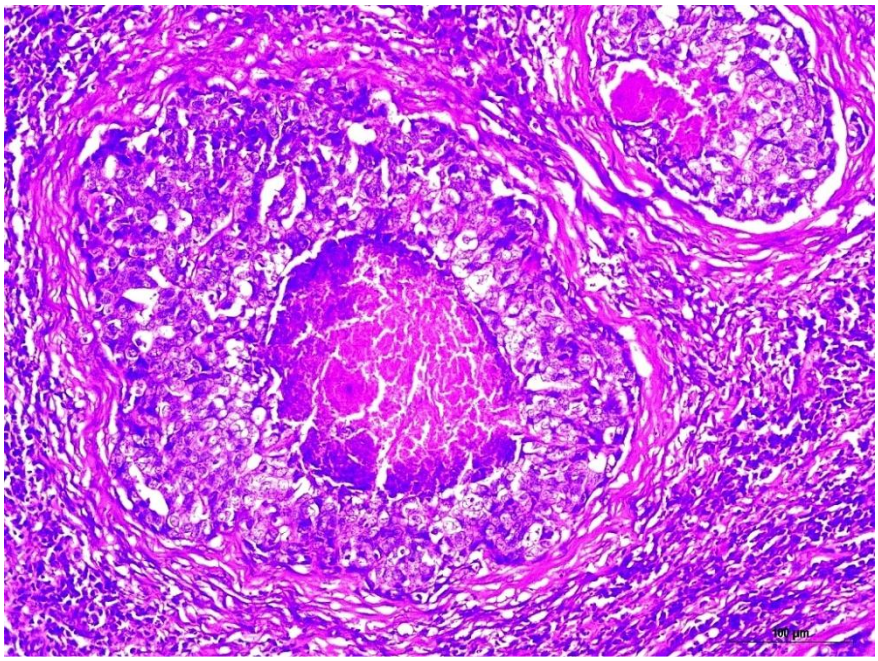

Plate 7. Comedocarcinoma. Necrotic areas in the centre of neoplastic cell aggregates (H&E x200)

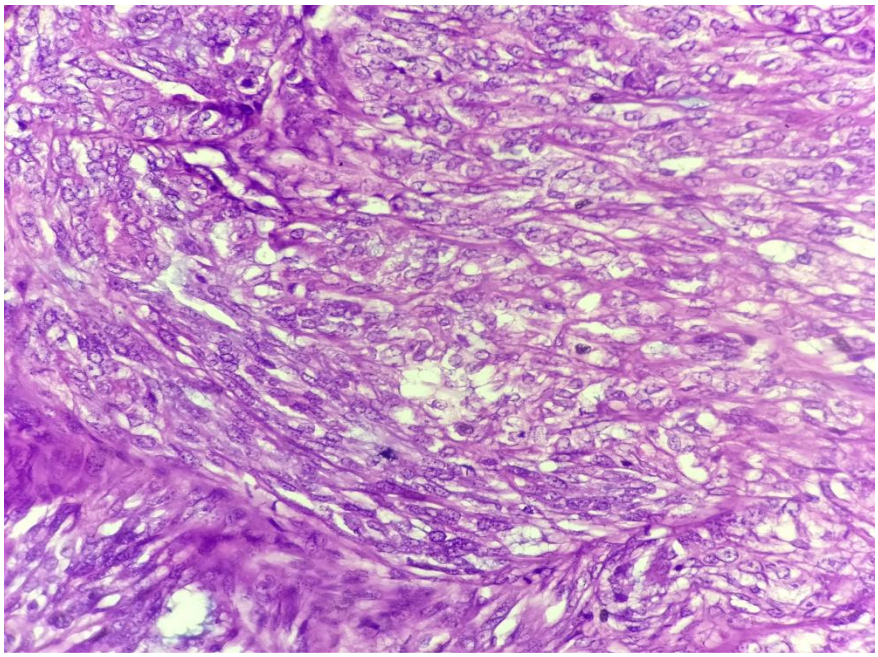

Plate 8. Spindle carcinoma. Numerous spindle shaped epithelial cells with elongated vesicular nuclei (H&E x200)

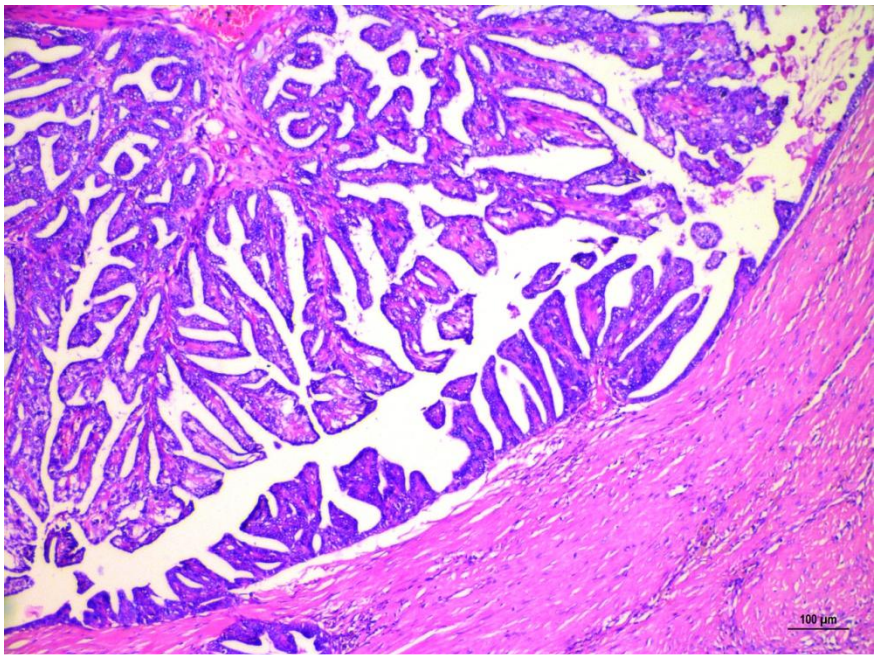

Plate 9. Tubulopapillary carcinoma. Tubular and papillary pattern of epithelial cell proliferation with delicate fibrovascular stalks (H&E x100)

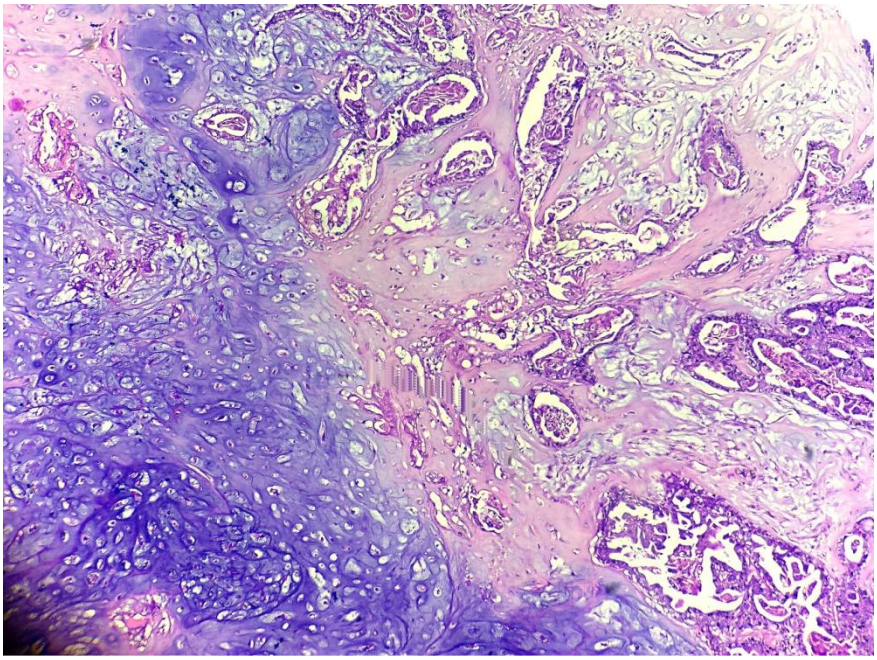

Plate 10. Carcinoma arising in benign mixed tumour. Focal areas of carcinomatous epithelial cells with marked cellular and nuclear pleomorphism and regions of cartilaginous metaplasia (H&E x100)

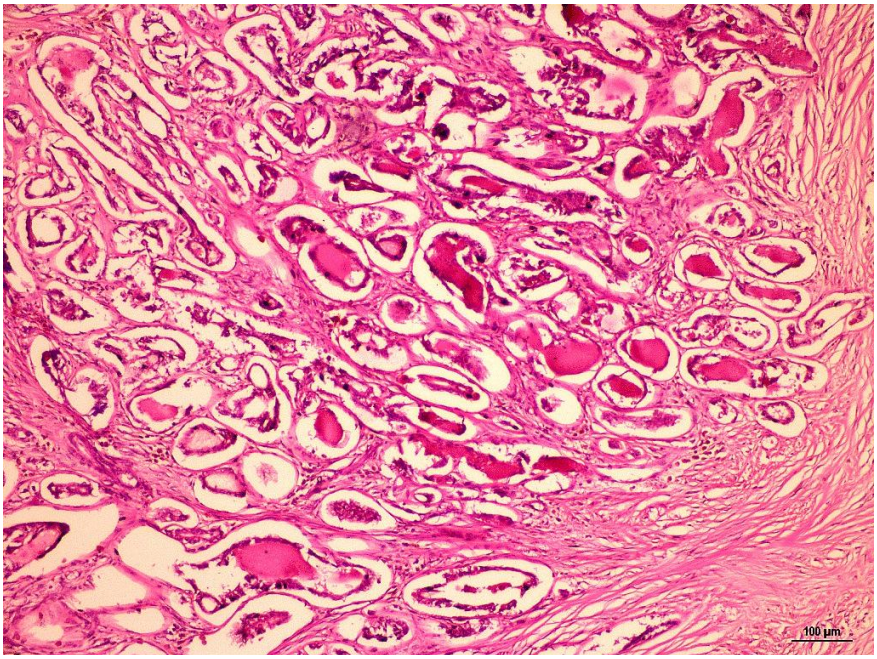

Plate 11. Fibroadenoma. Extensive stroma of dense fibrous connective tissue and tubules lined by cuboidal or columnar cells (H&E x100)

Plate 12. Squamous cell carcinoma. Ulcerated mass on digit

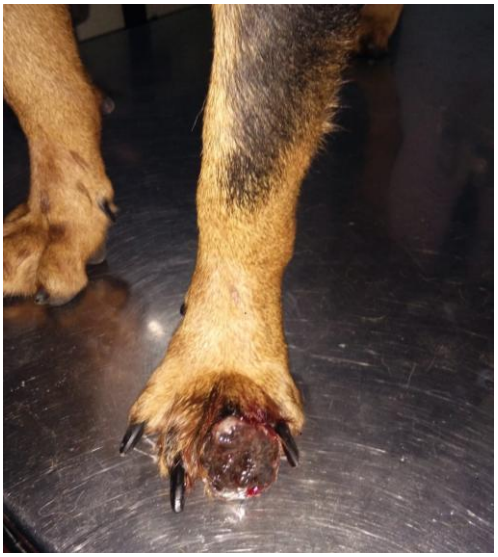

Plate 13. Melanocytoma. Digit

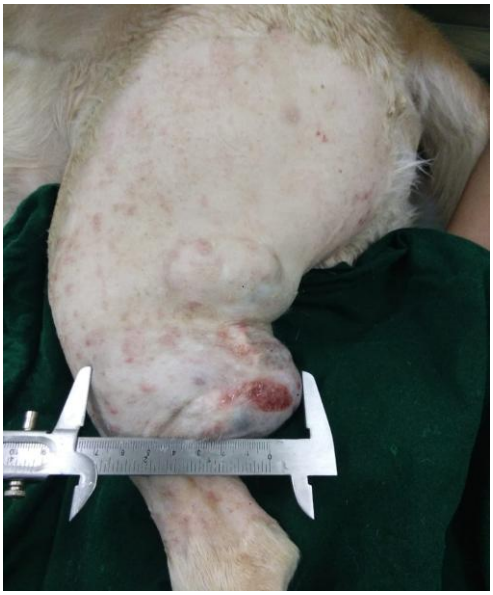

Plate 14.A. Myxosarcoma. Left thigh

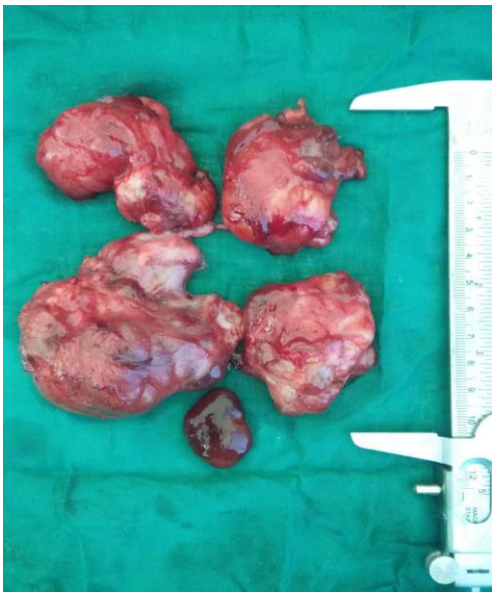

Plate 14.B. Myxosarcoma. Multiple tumour masses removed

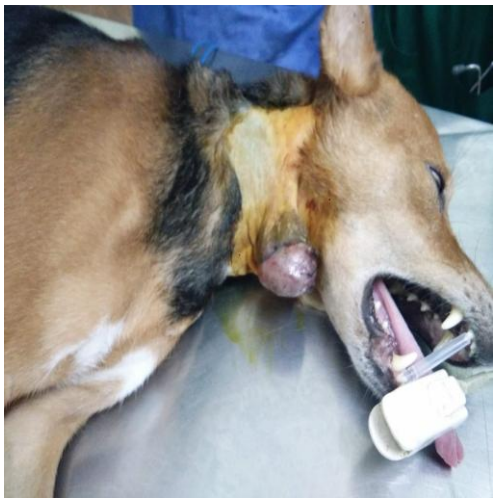

Plate 15.A. Trichoblastoma. Neck

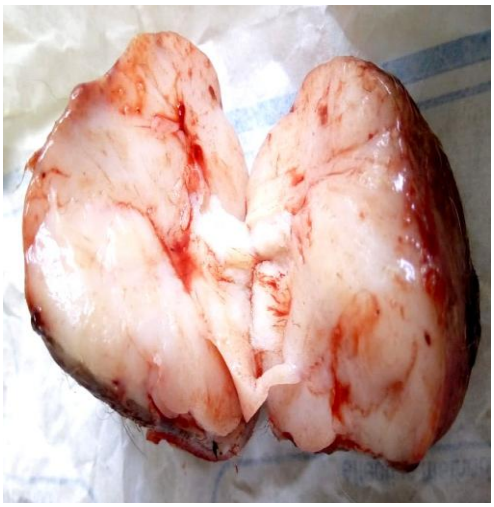

Plate 15.B. Trichoblastoma. Cut surface with grayish white colour

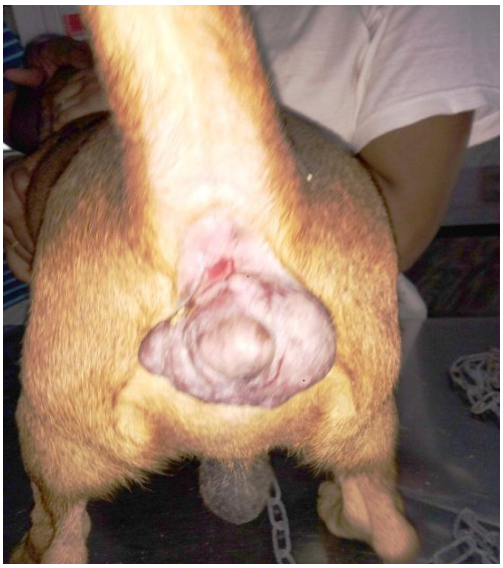

Plate 16.A. Hepatoid gland adenoma

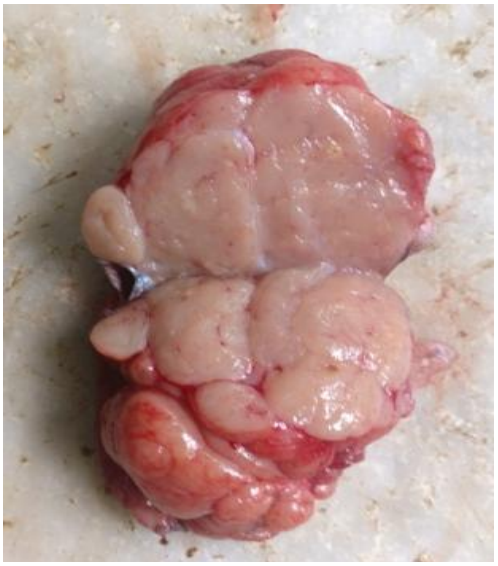

Plate 16.B. Hepatoid gland adenoma. Cut surface

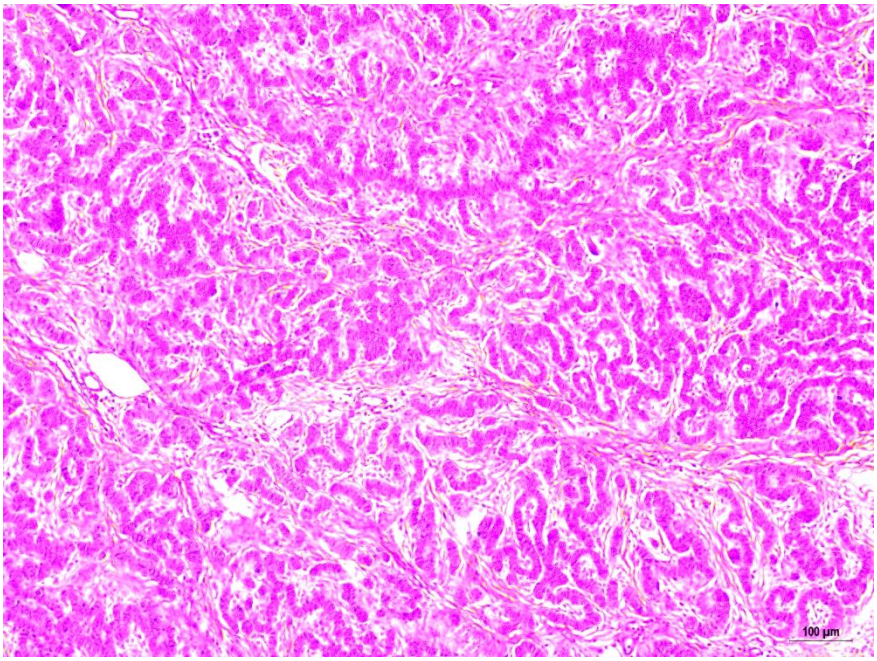

Plate 17. Trichoblastoma. Ribbon type long cords of branching and anastomosing cells (H&E x100)

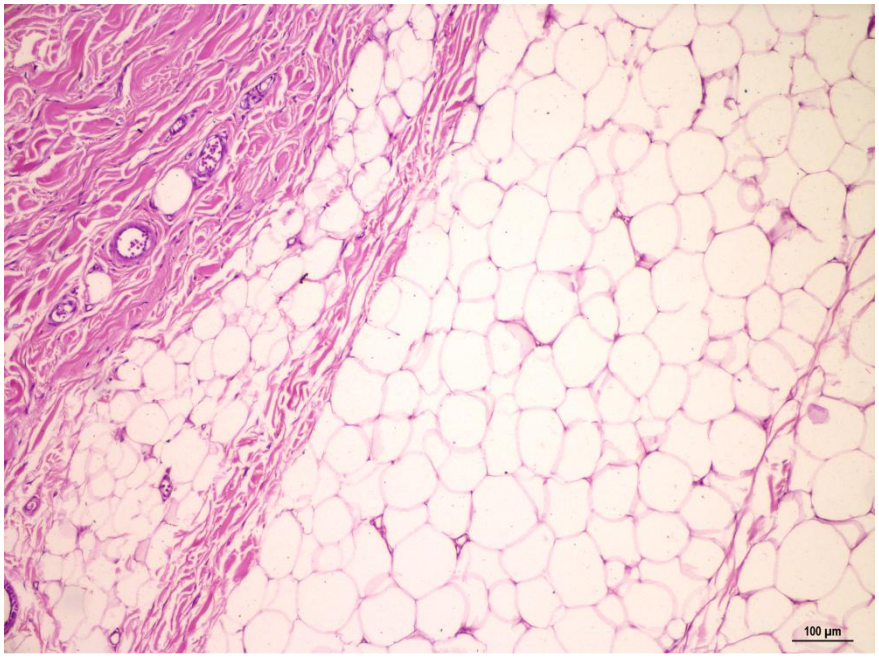

Plate 18. Lipoma. Neoplastic adipocytes with single, clear and large cytoplasmic vacuole with the nuclei compressed to the periphery (H&E x100)

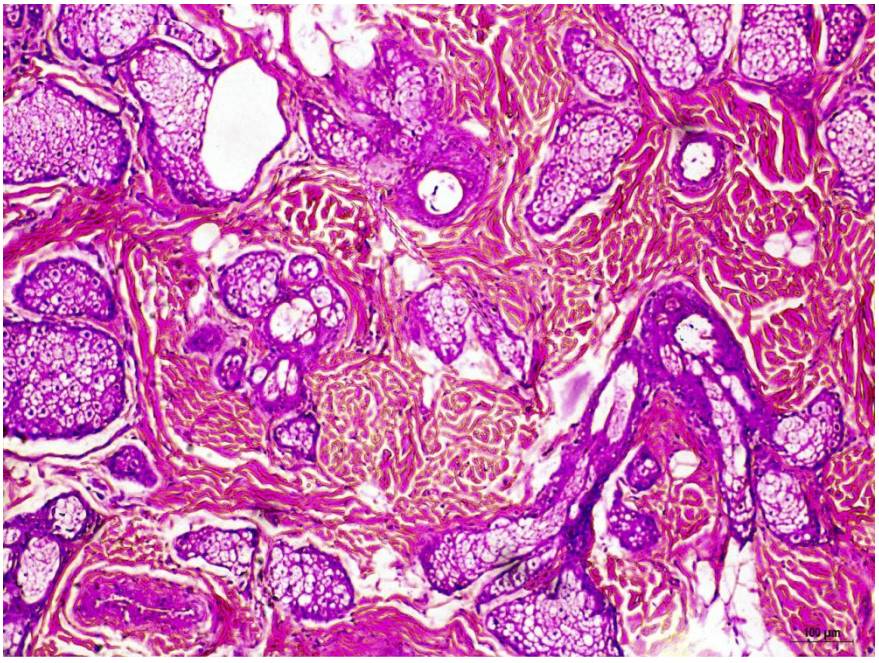

Plate 19. Sebaceous adenoma. Neoplastic cells arranged in multiple lobules and separated by connective tissue trabeculae (H&E x100)

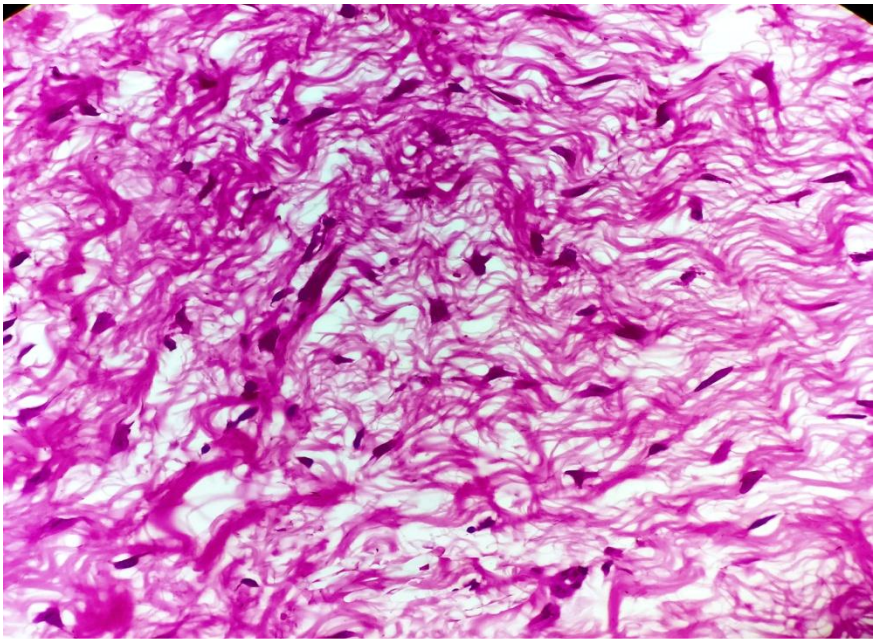

Plate 20. Fibroma. Proliferation of spindle-shaped and stellate fibroblasts and presence of abundant collagenous stroma (H&E x200)

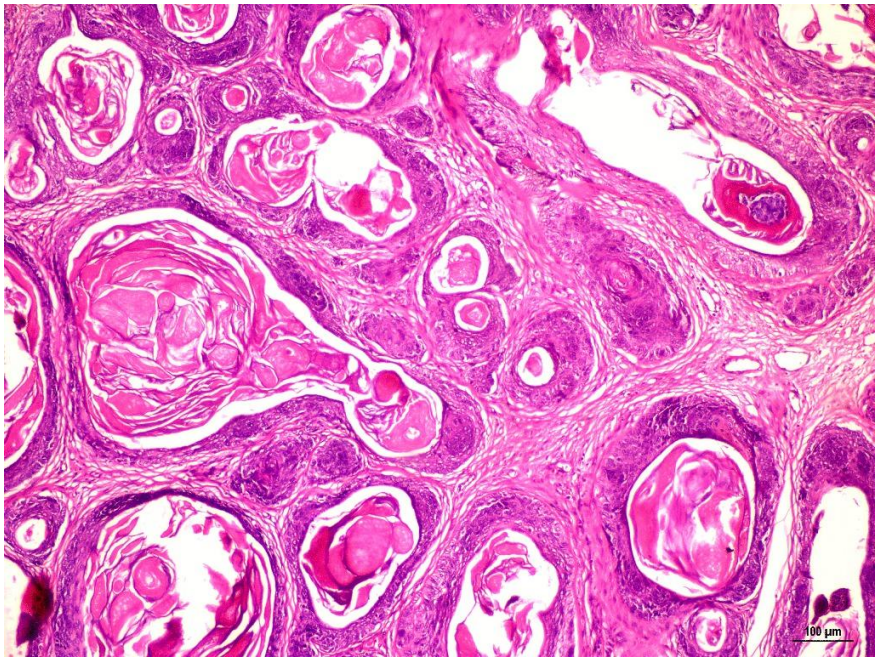

Plate 21. Trichoepithelioma. Cysts layered by neoplastic epithelial cells and filled with keratinous debris (H&E x100)

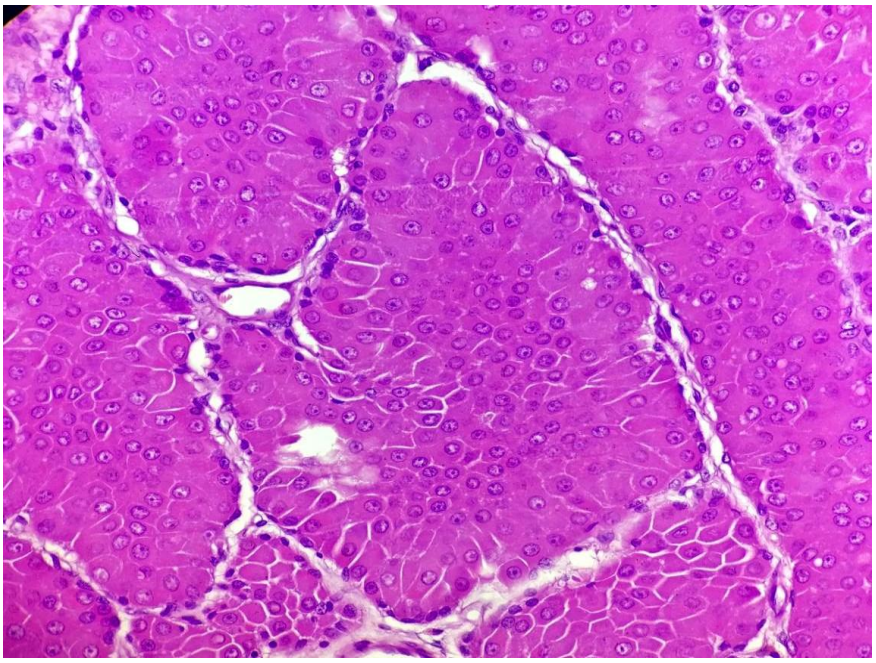

Plate 22. Hepatoid gland adenoma. Cords of cells with similarity to normal hepatocytes (H&E x200)

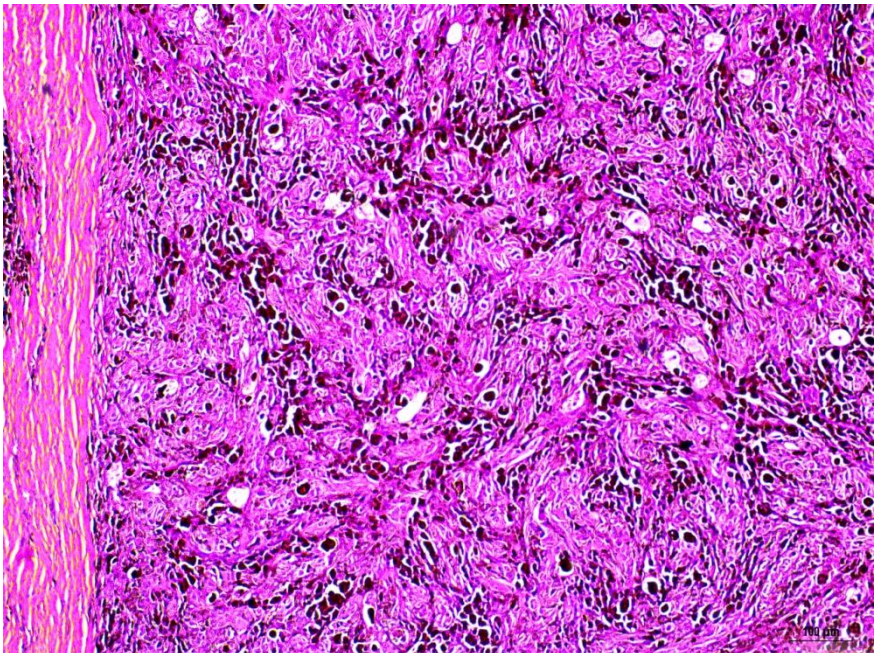

Plate 23. Melanocytoma. Presence of abundant quantity of melanin pigment in the cytoplasm of neoplastic melanocytes (H&E x100)

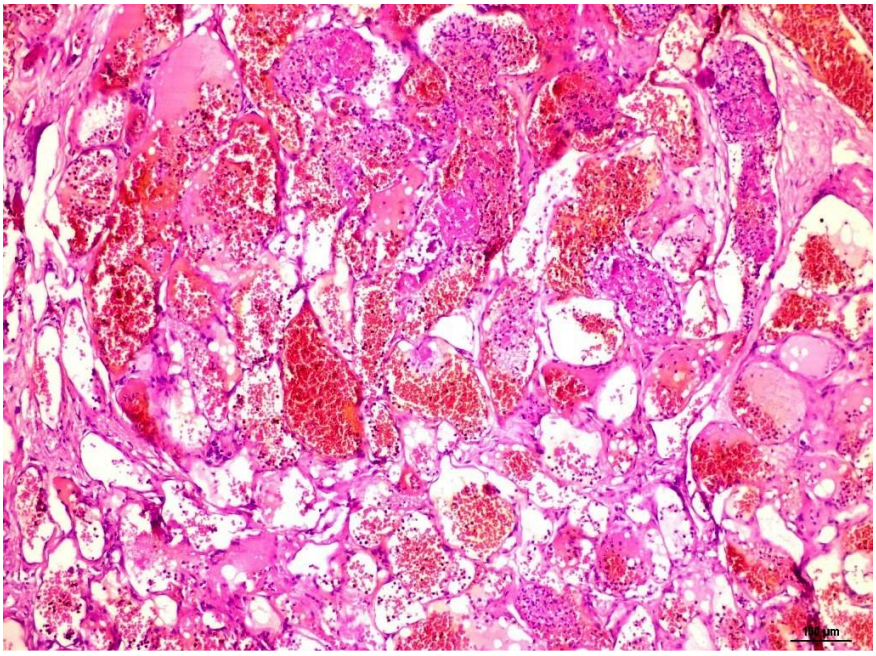

Plate 24. Haemangioma. Differently sized vascular spaces filled with erythrocytes and lined by single layer of endothelial cells (H&E x100)

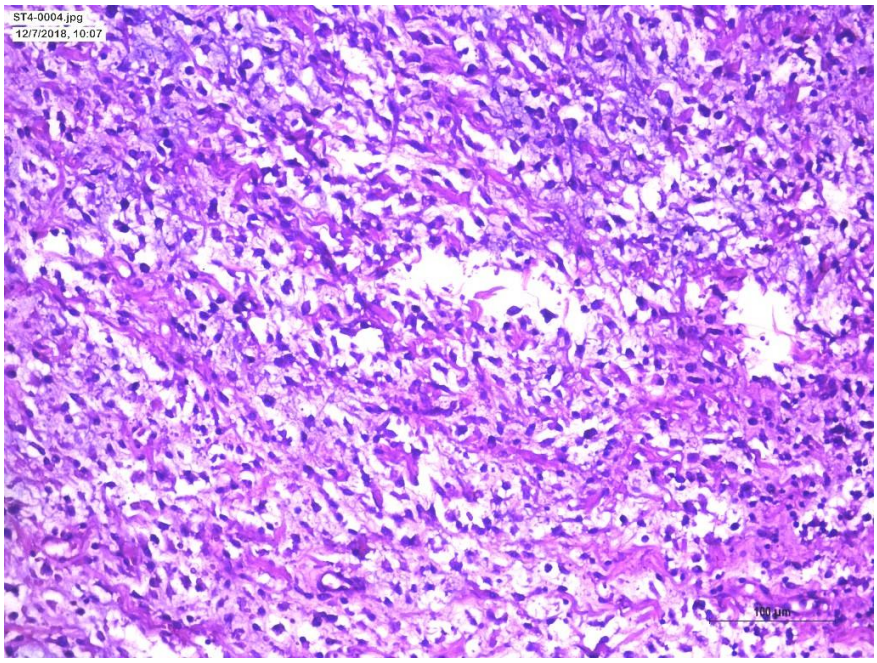

Plate 25. Myxosarcoma. Proliferation of spindle shaped fibroblasts in an abundant myxoid matrix (H&E x200)

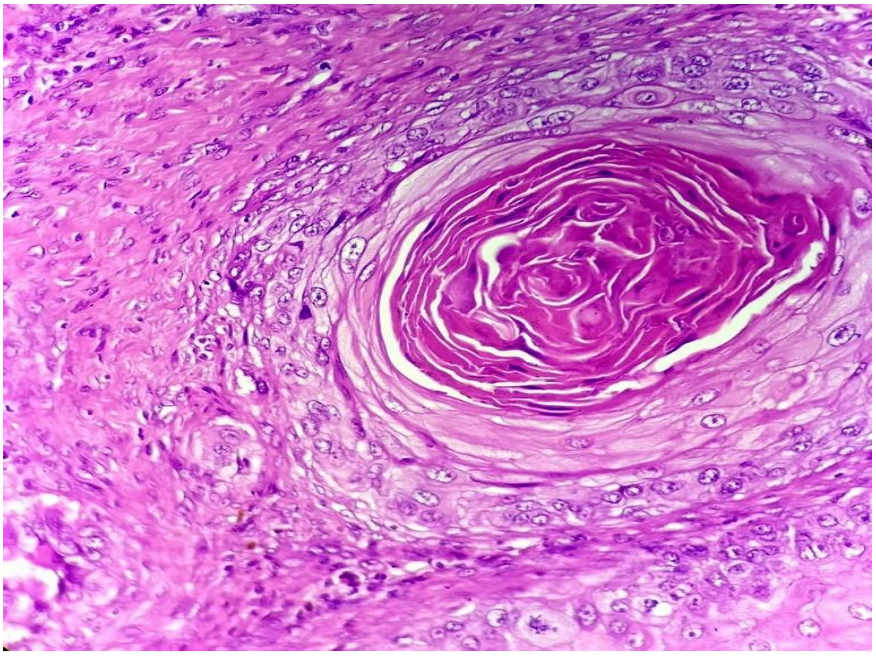

Plate 26. Well differentiated squamous cell carcinoma with cords of neoplastic cells and formation of distinct keratin pearls (H&E x200)

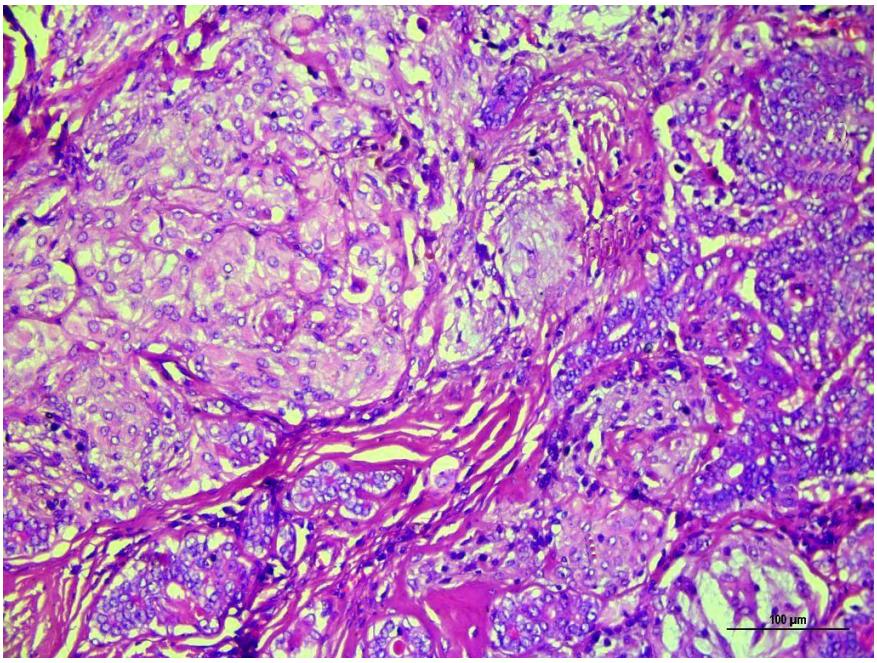

Plate 27. Less differentiated squamous cell carcinoma with polygonal and smaller squamous cells without keratin pearls (H&E x200)

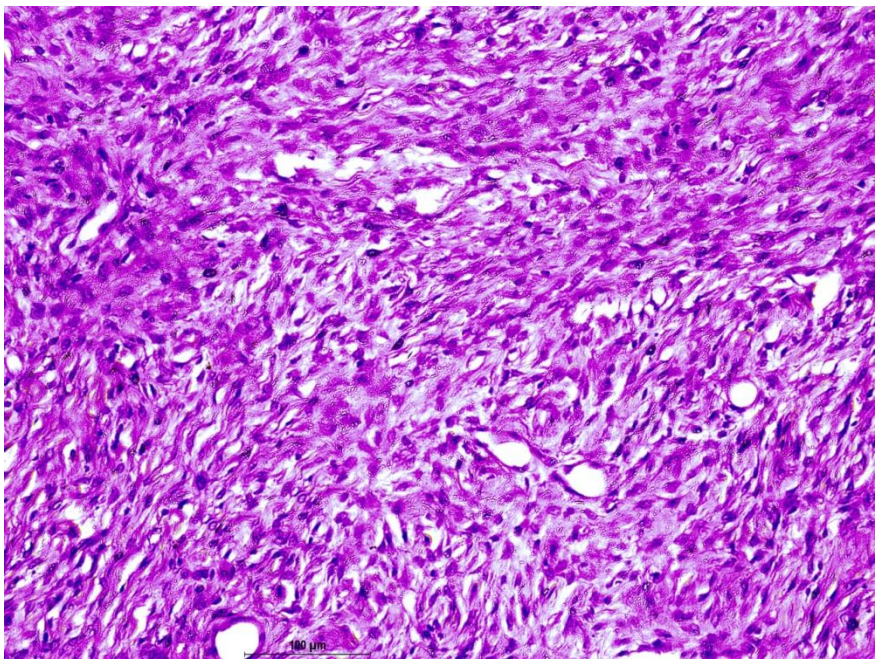

Plate 28. Fibrosarcoma. Spindle shaped tumour cells and collagen fibers arranged in herringbone pattern (H&E x200)

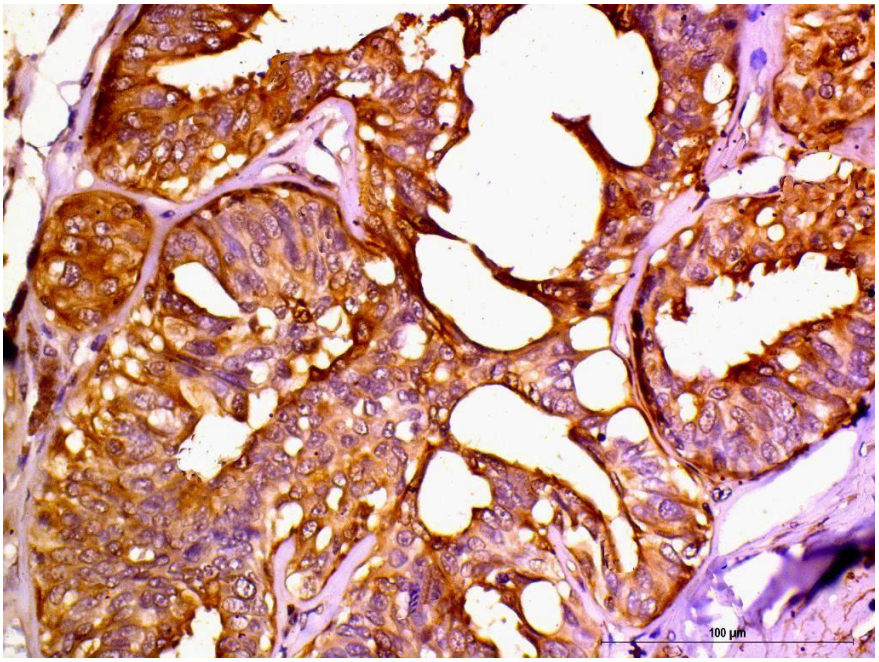

Plate 29. Ductal carcinoma. Strong expression of mTOR (IHC x400)

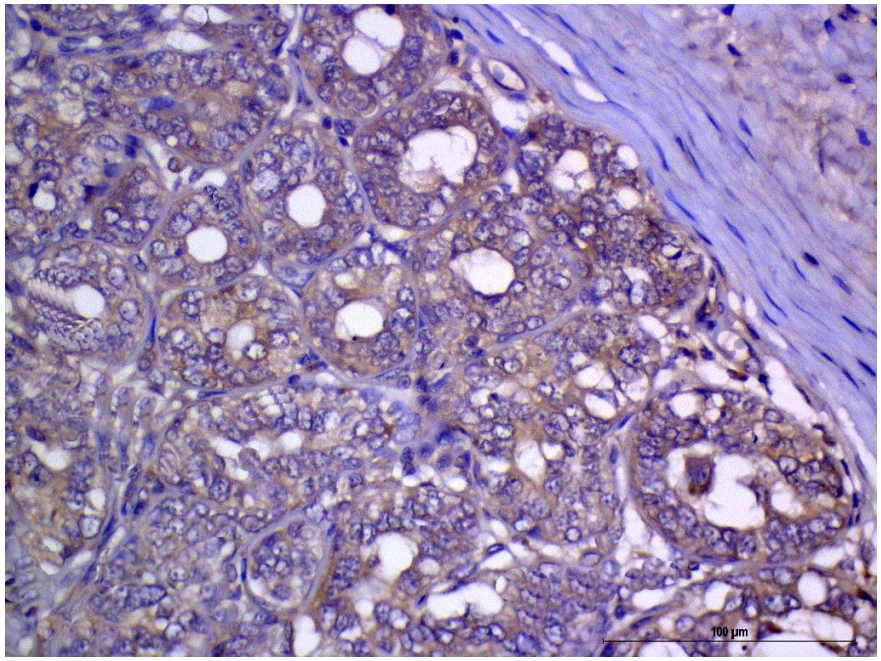

Plate 30. Ductal carcinoma. Moderate expression of mTOR (IHC x400)

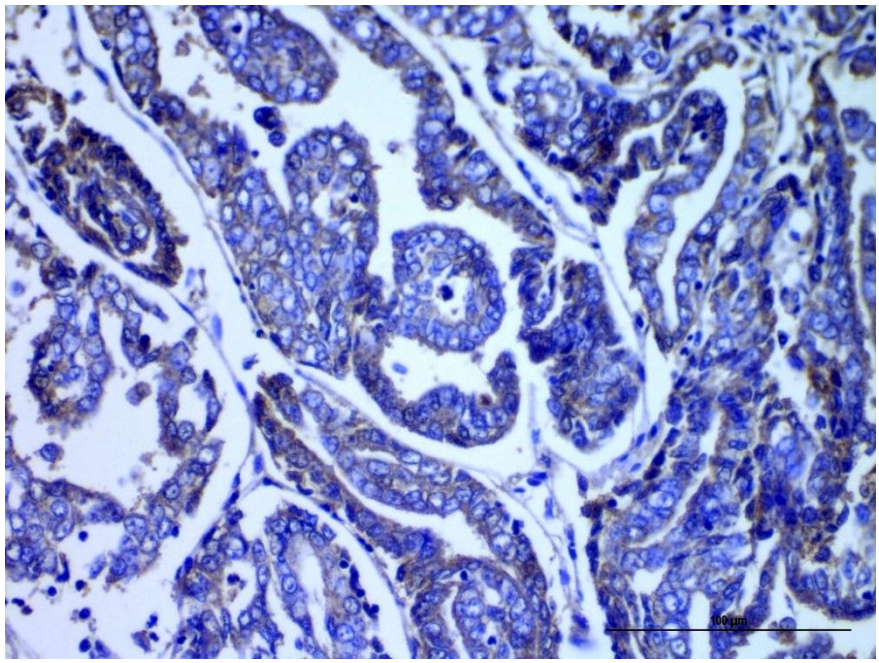

Plate 31. Ductal carcinoma. Weak expression of mTOR (IHC x400)

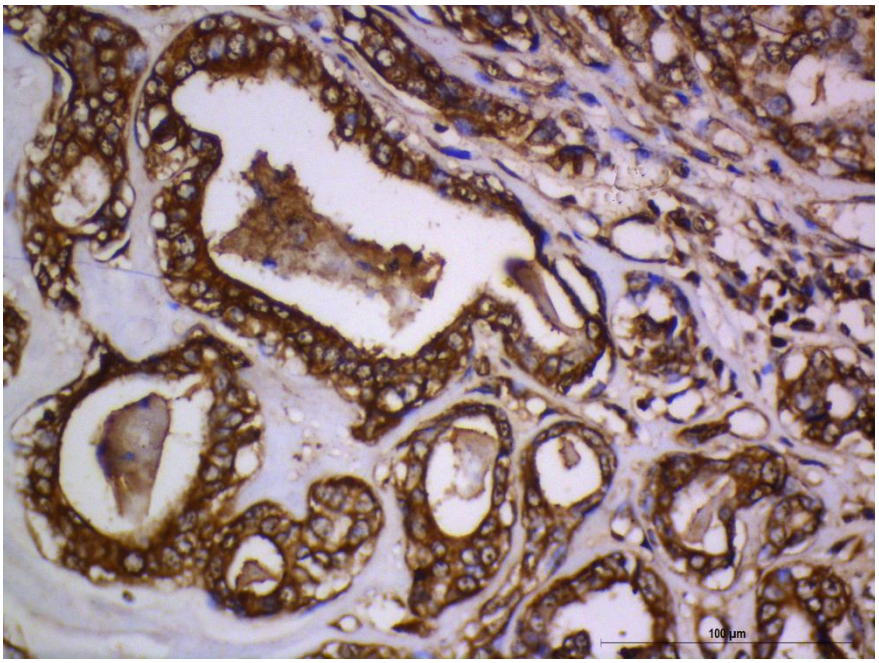

Plate 32. Carcinoma arising in benign mixed tumour. Strong expression of mTOR (IHC x400)

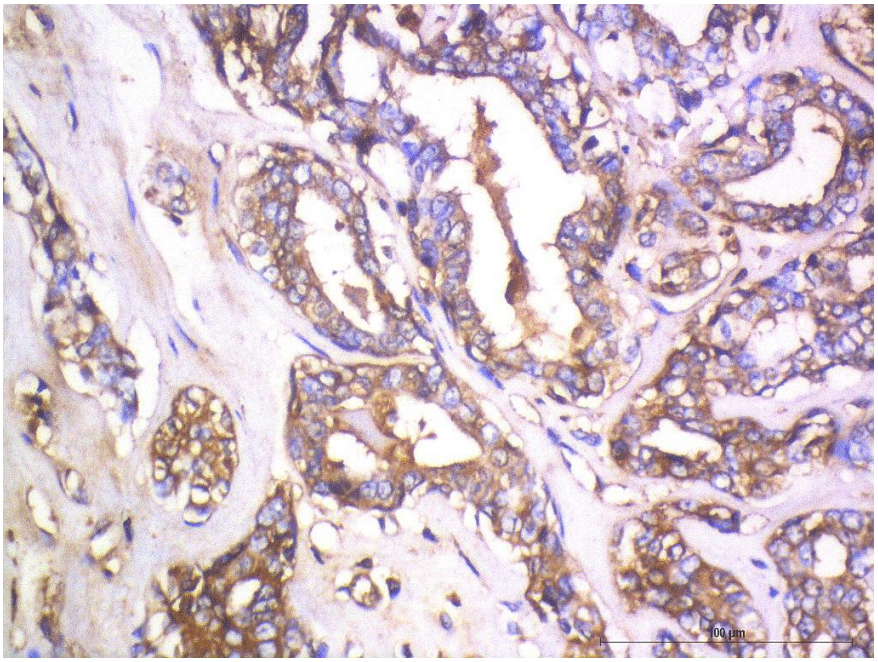

Plate 33. Carcinoma arising in benign mixed tumour. mTOR moderate expression (IHC x400)

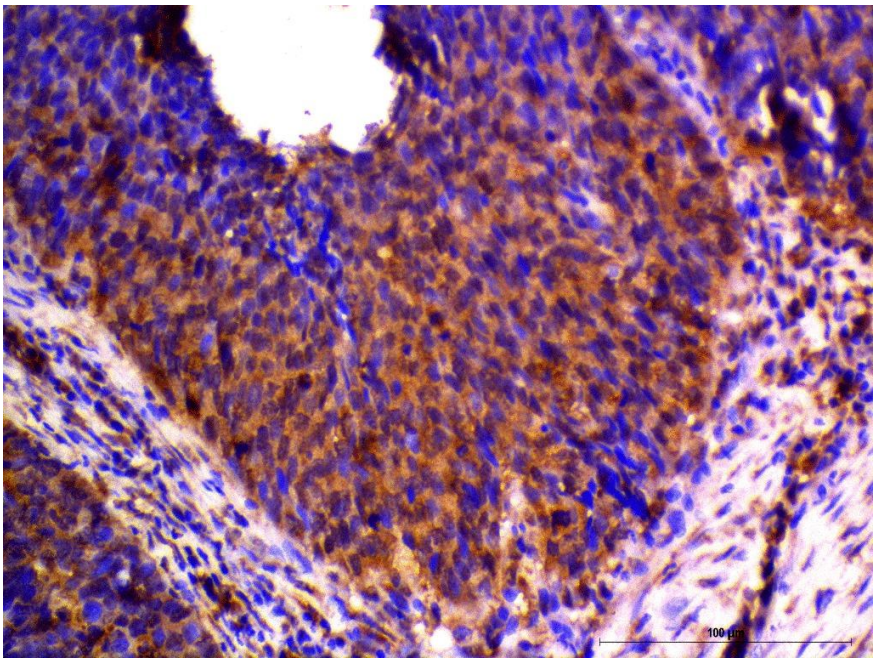

Plate 34: Comedocarcinoma. mTOR strong expression (IHC x400)

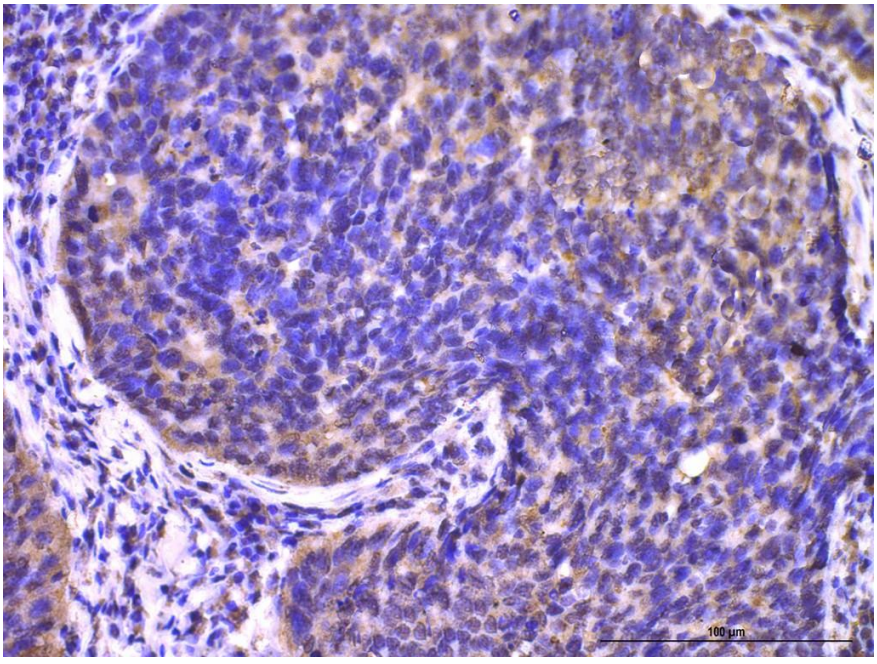

Plate 35. Solid carcinoma. mTOR moderate expression (IHC x400)

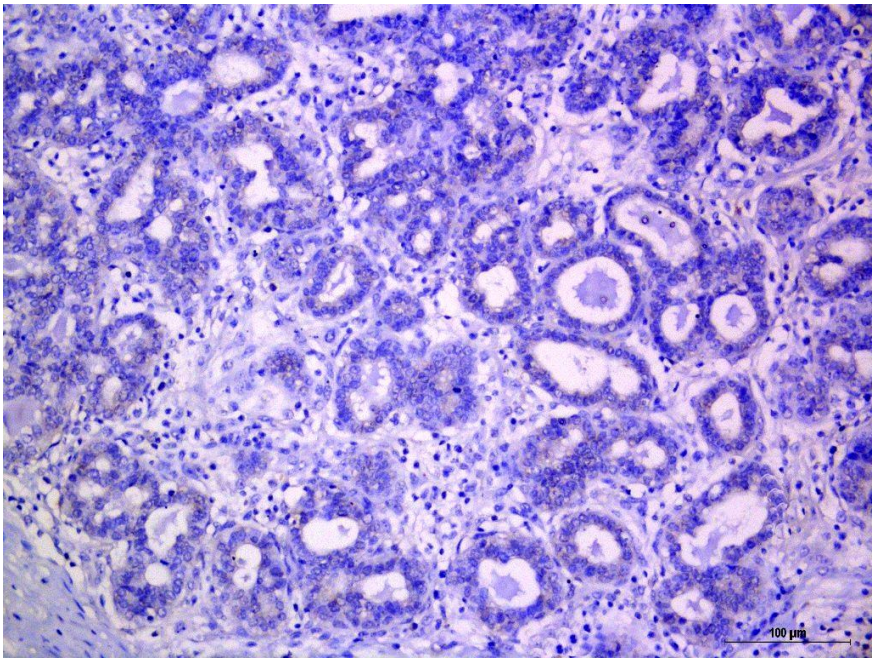

Plate 36. Fibroadenoma. mTOR weak expression (IHC x200)

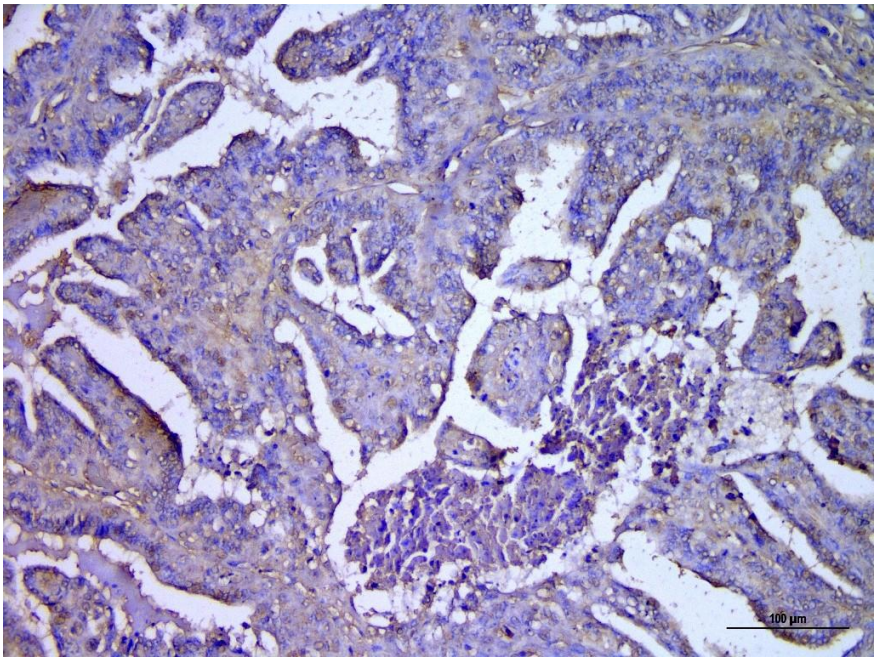

Plate 37. Tubulopapillary carcinoma. mTOR moderate expression (IHC x200)

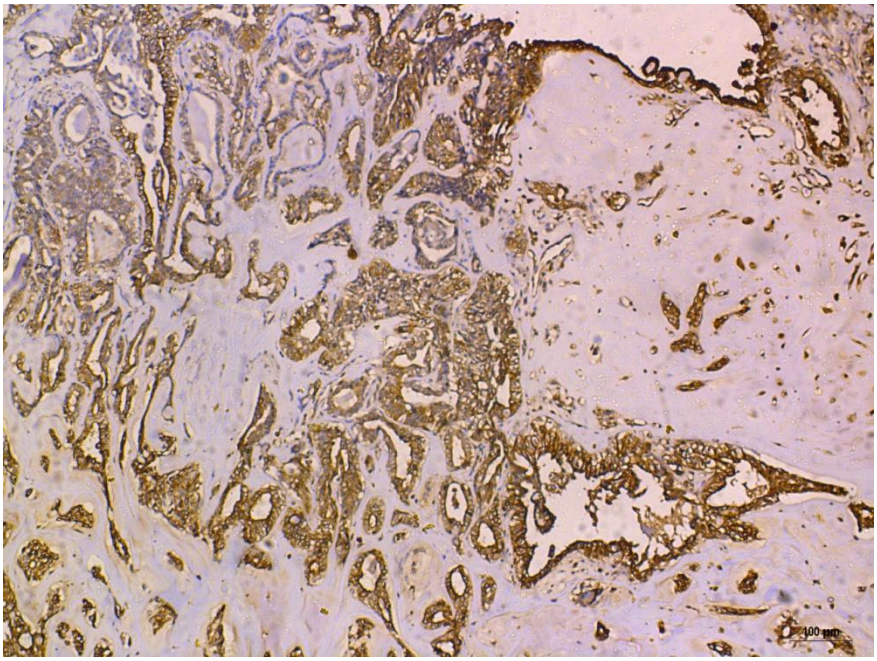

Plate 38. Carcinoma arising in benign mixed tumour. DEPTOR strong expression (IHC x100)

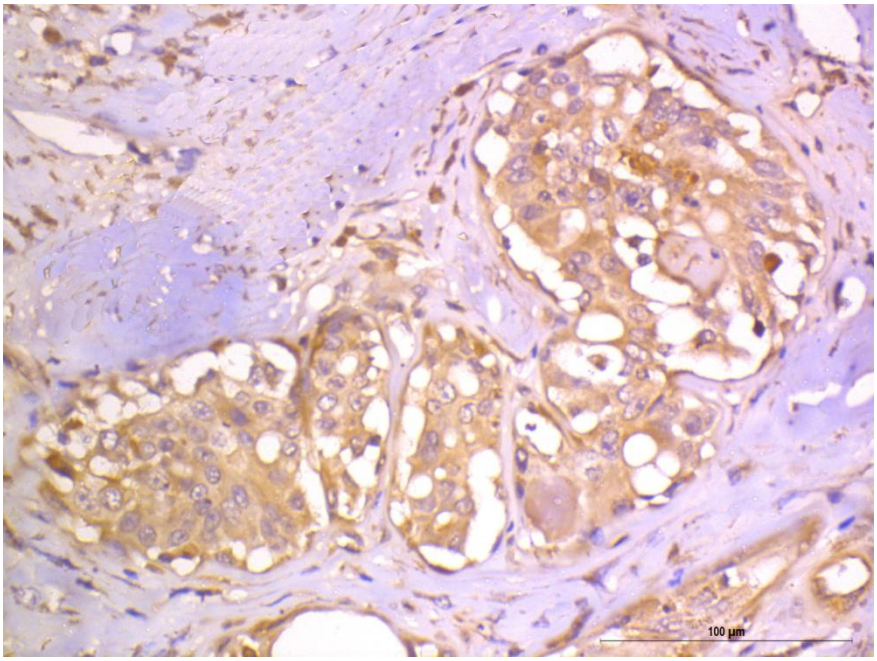

Plate 39. Carcinoma arising in benign mixed tumour. DEPTOR moderate expression (IHC x400)

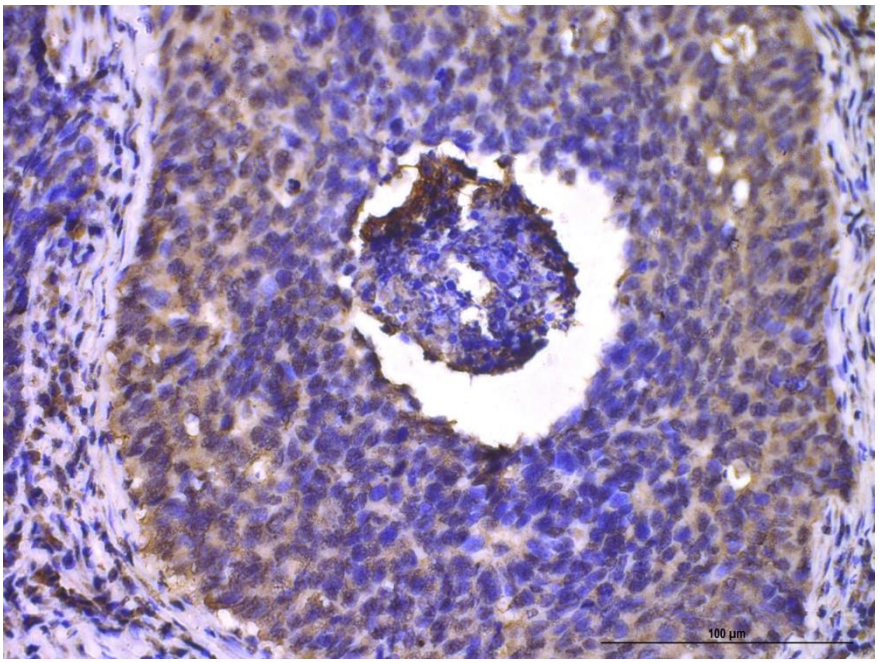

Plate 40. Comedocarcinoma. DEPTOR moderate expression (IHC x400)

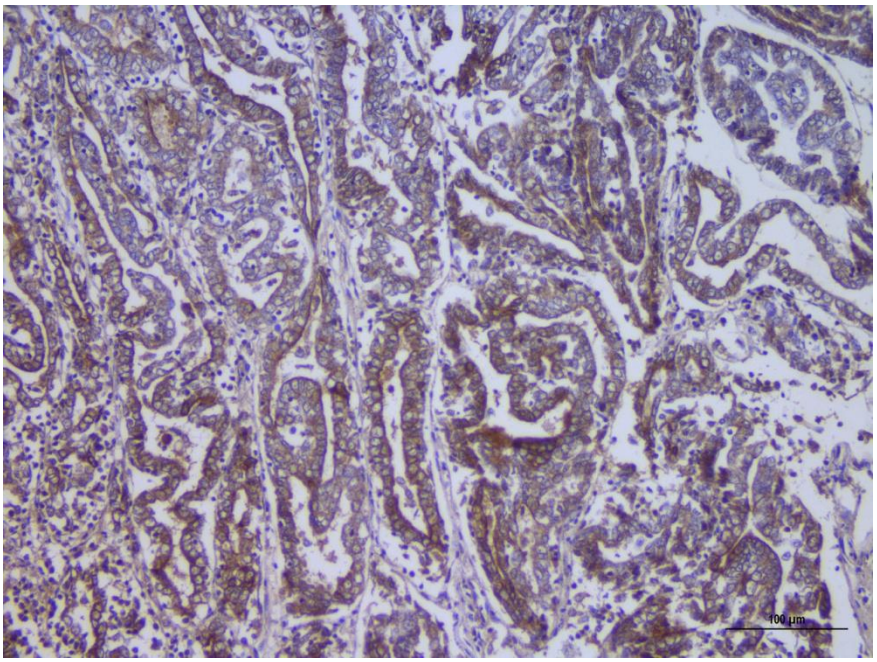

Plate 41. Ductal carcinoma. DEPTOR moderate expression (IHC x200)

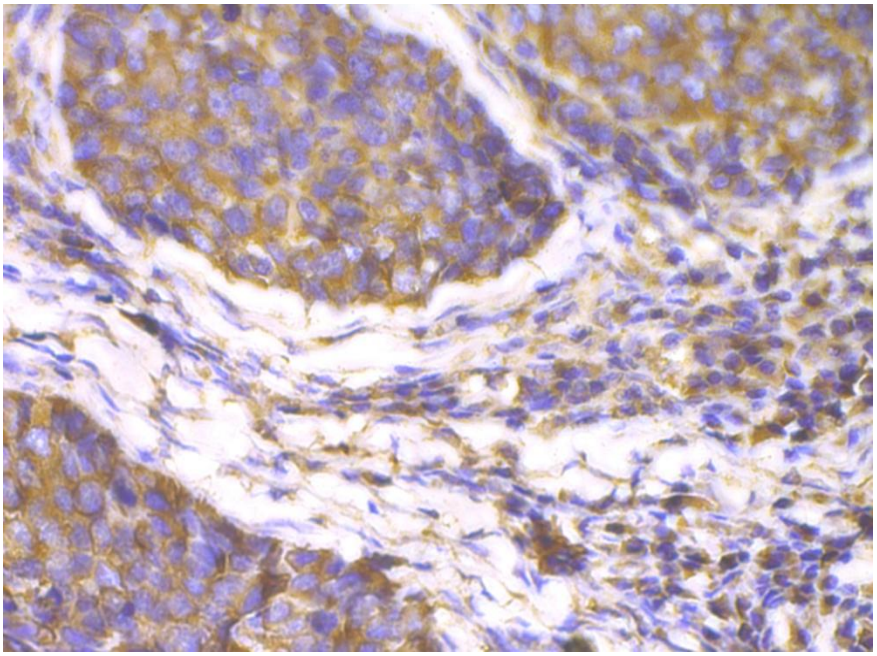

Plate 42. Solid carcinoma. DEPTOR moderate expression (IHC x400)

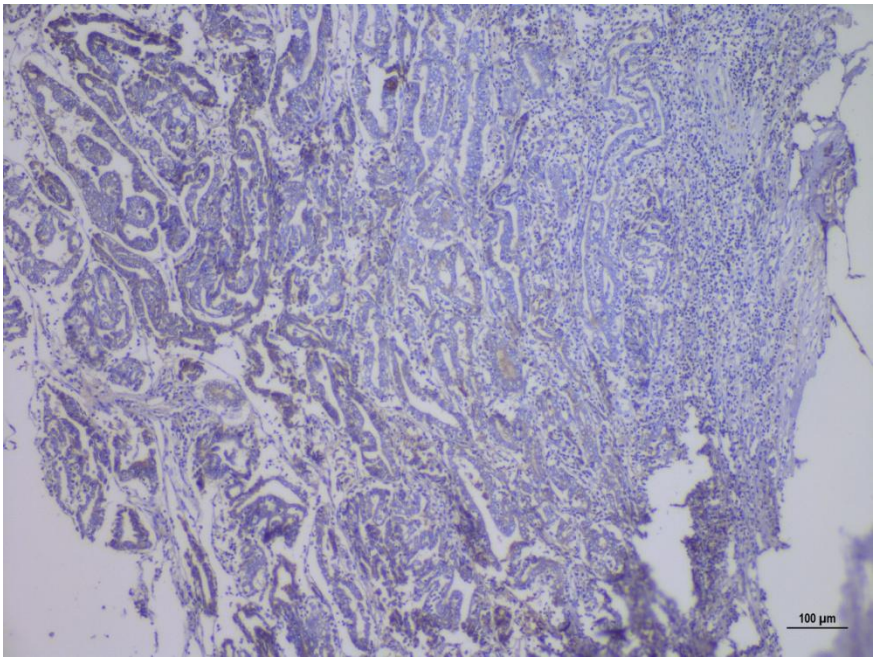

Plate 43. Ductal carcinoma. DEPTOR weak expression (IHC x100)

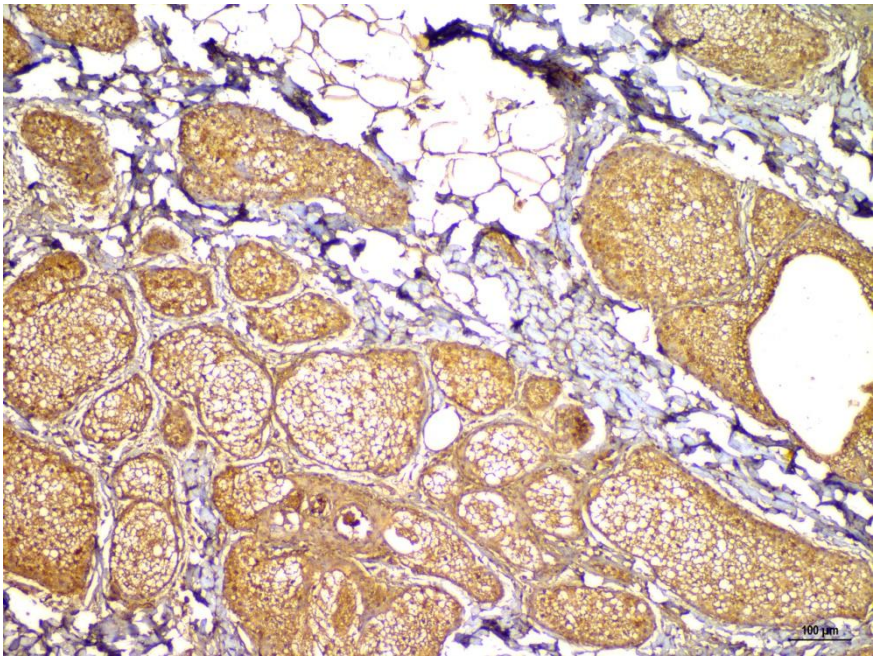

Plate 44. Sebaceous adenoma. mTOR strong expression (IHC x100)

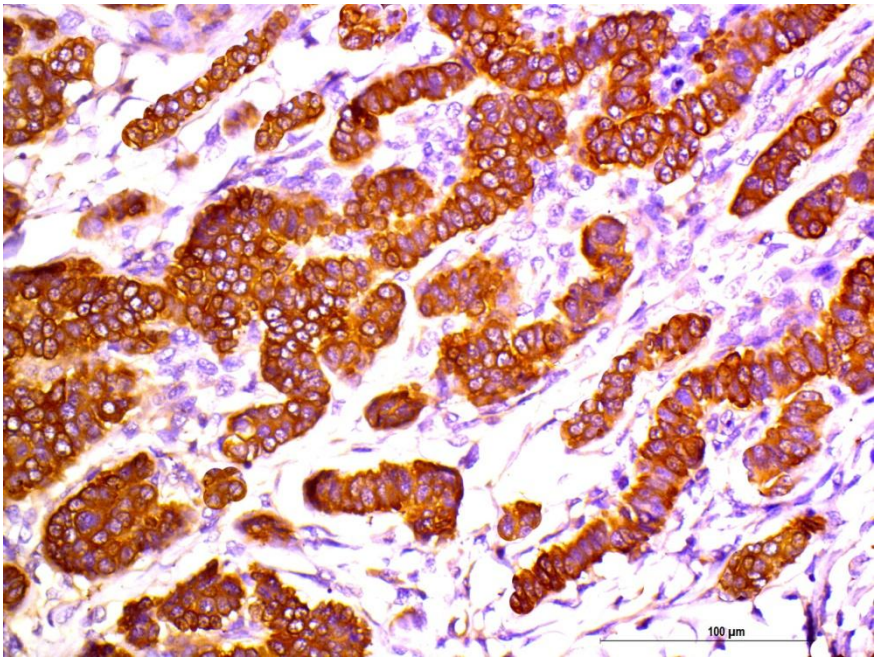

Plate 45. Trichoblastoma. mTOR strong expression (IHC x400)

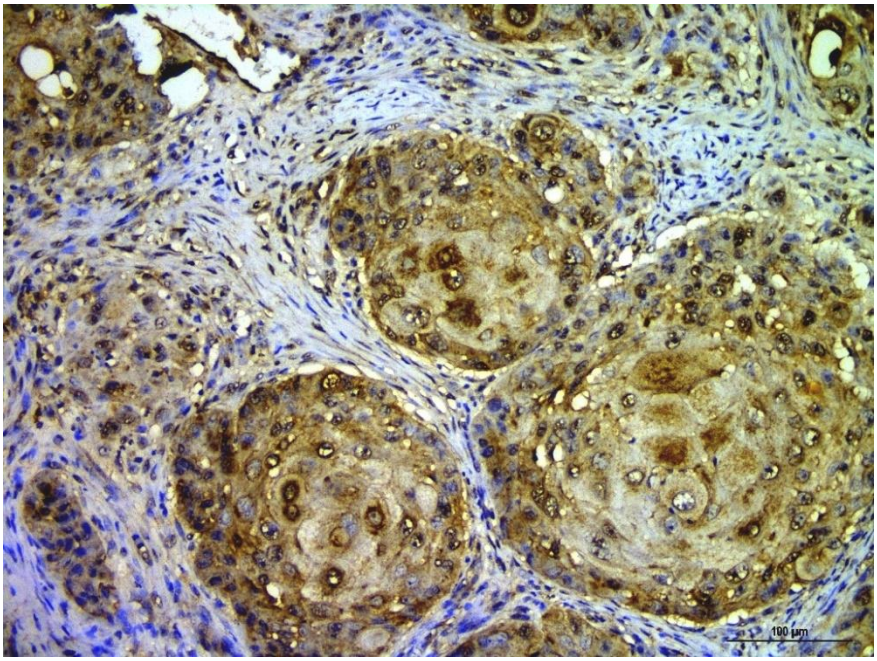

Plate 46. Squamous cell carcinoma. mTOR strong expression (IHC x200)

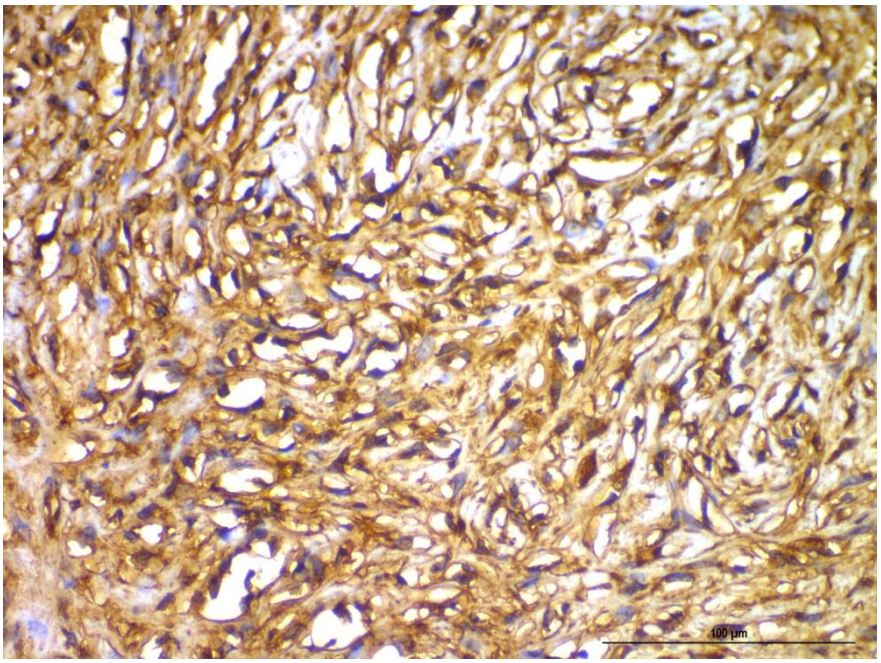

Plate 47. Fibrosarcoma. mTOR strong expression (IHC x400)

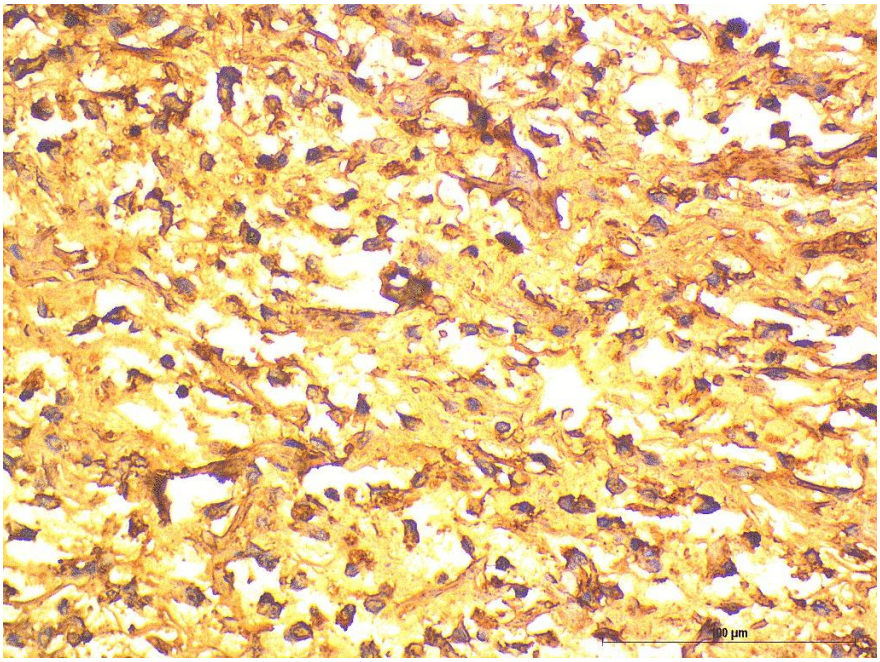

Plate 48. Myxosarcoma. mTOR moderate expression (IHC x400)

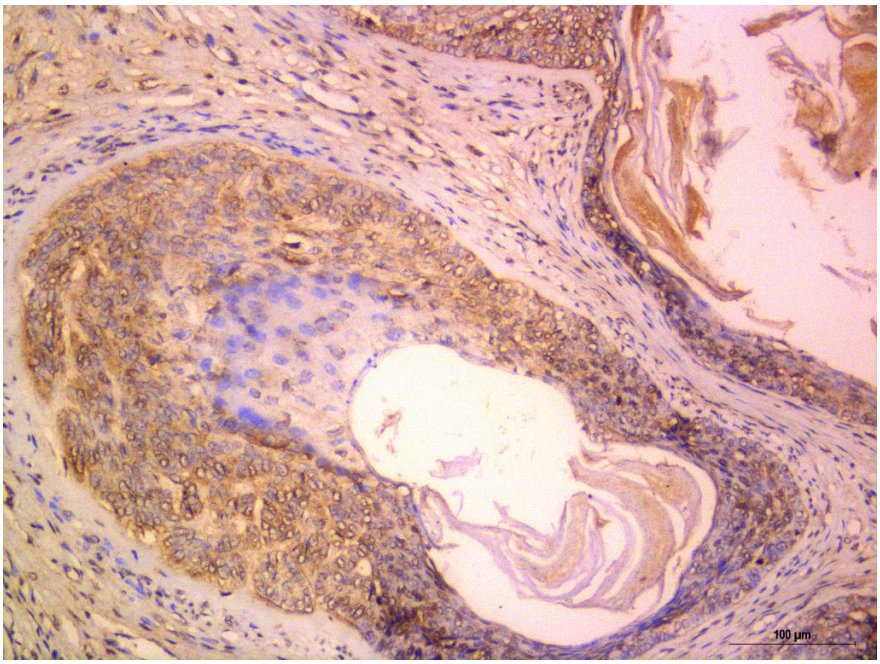

Plate 49. Trichoepithelioma. mTOR moderate expression (IHC x200)

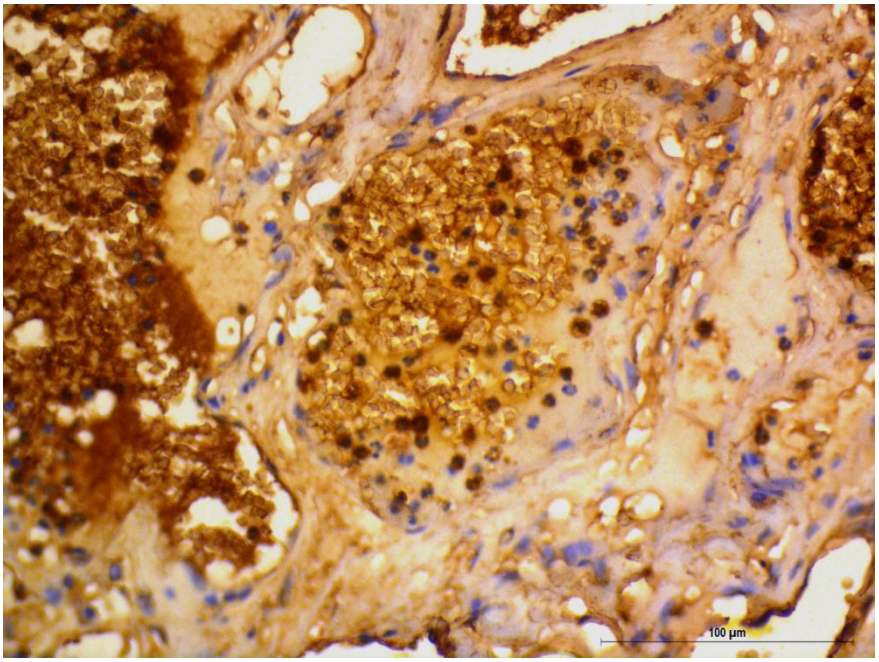

Plate 50. Haemangioma. mTOR moderate expression (IHC x400)

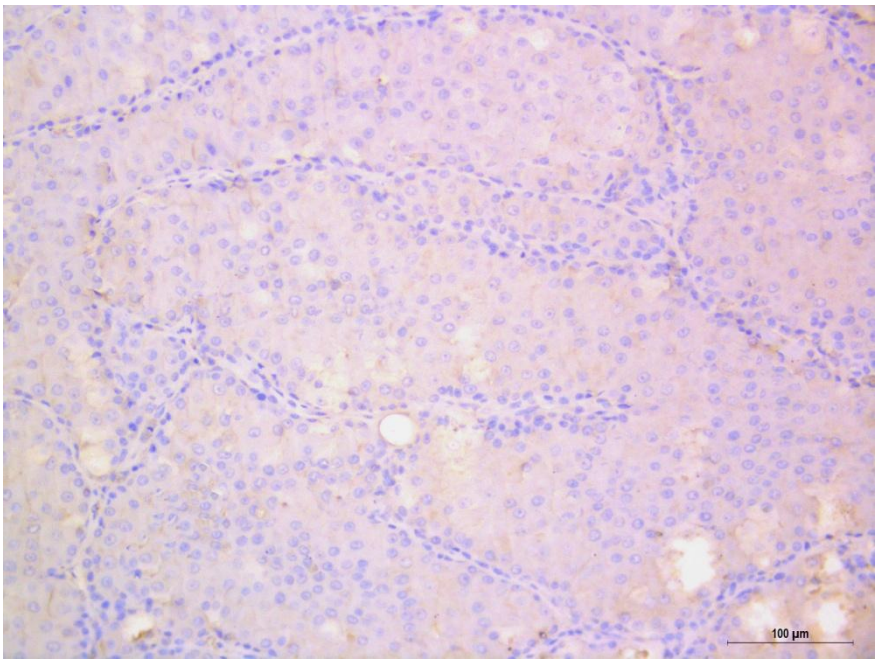

Plate 51. Hepatoid adenoma. mTOR weak expression (IHC x200)

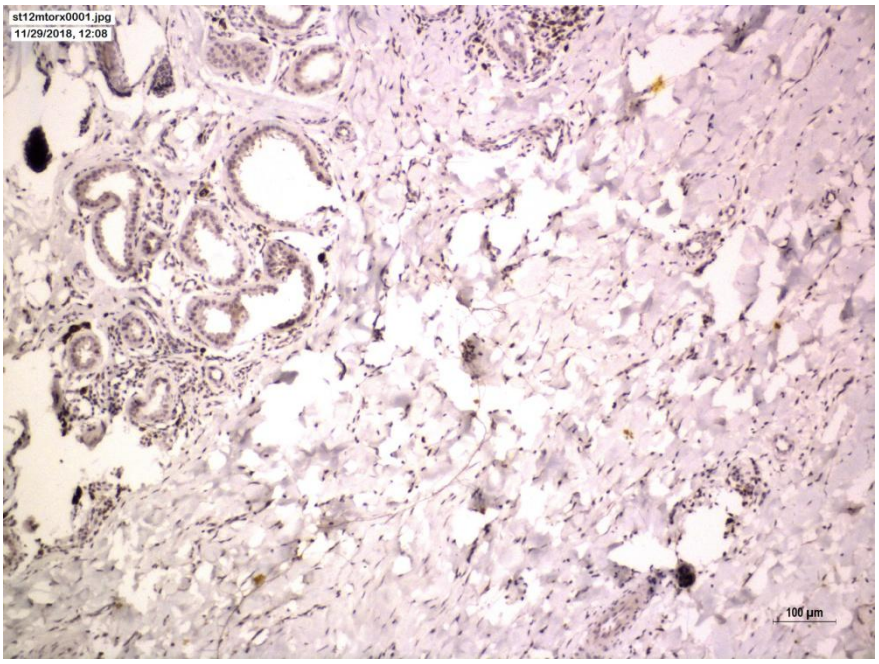

Plate 52. Melanocytoma. mTOR weak expression (IHC x100)

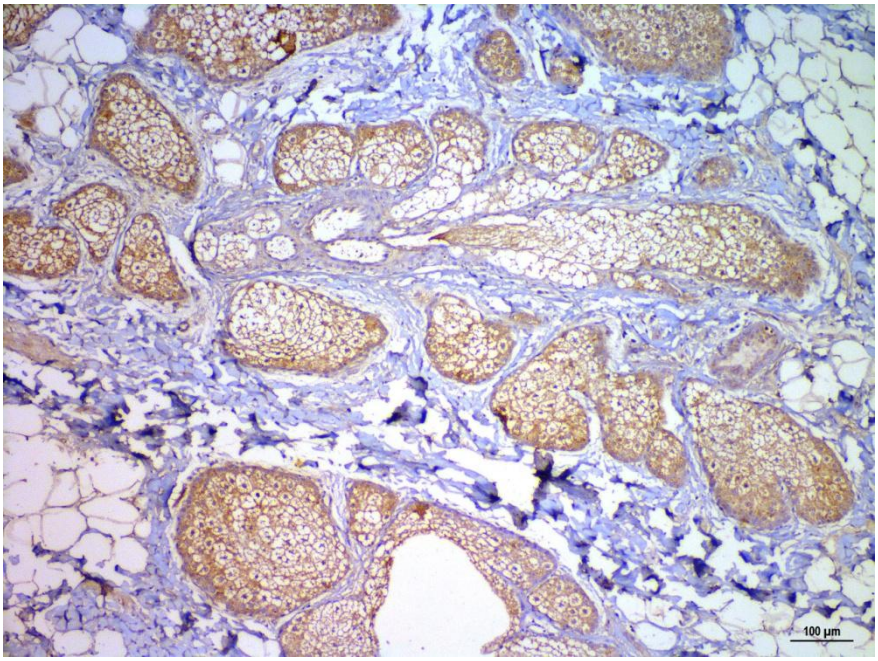

Plate 53. Sebaceous adenoma. DEPTOR moderate expression (IHC x100)

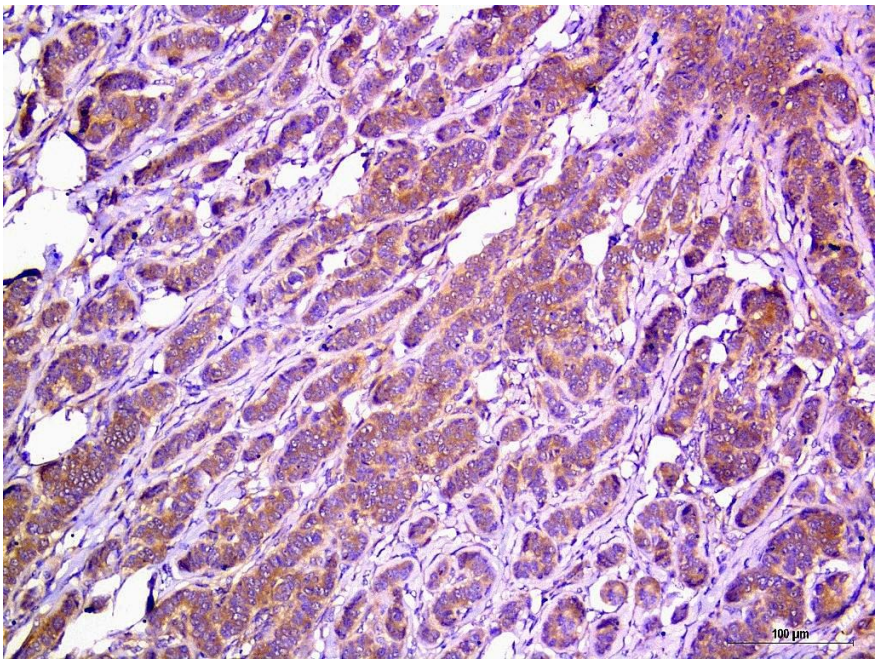

Plate 54. Trichoblastoma. DEPTOR moderate expression (IHC x200)

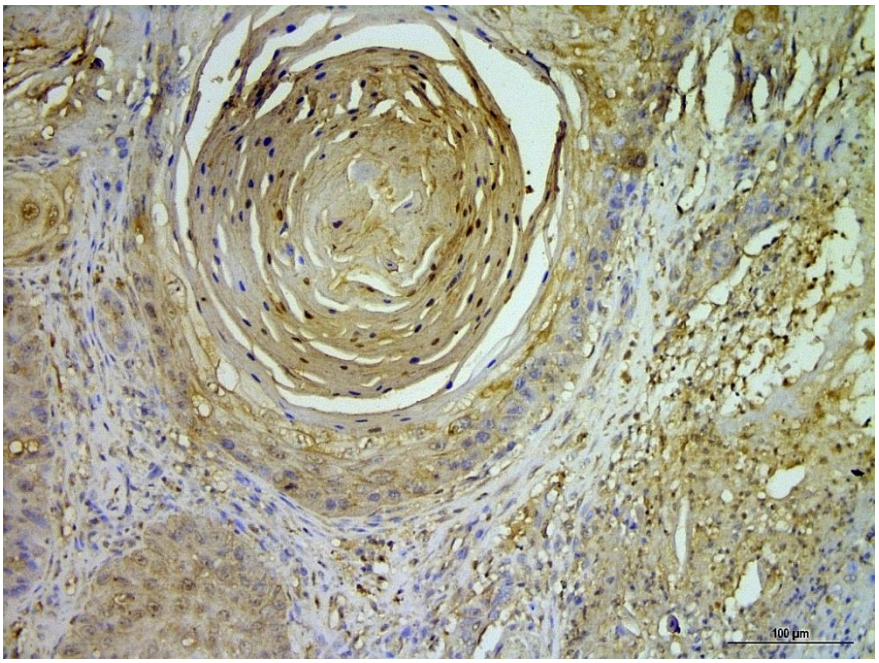

Plate 55. Squamous cell carcinoma. DEPTOR moderate expression (IHC x200)

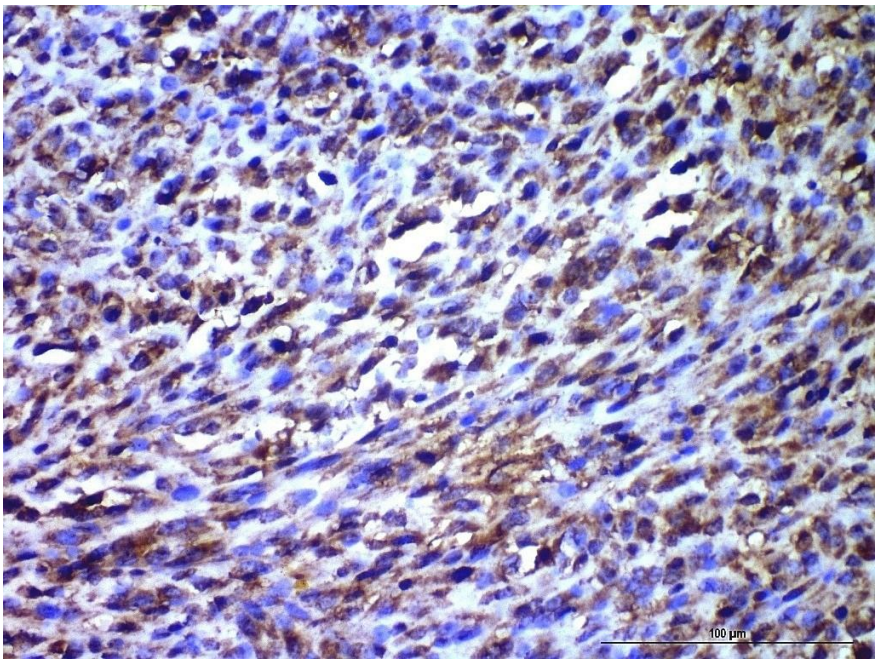

Plate 56. Myxosarcoma. DEPTOR moderate expression (IHC x400)

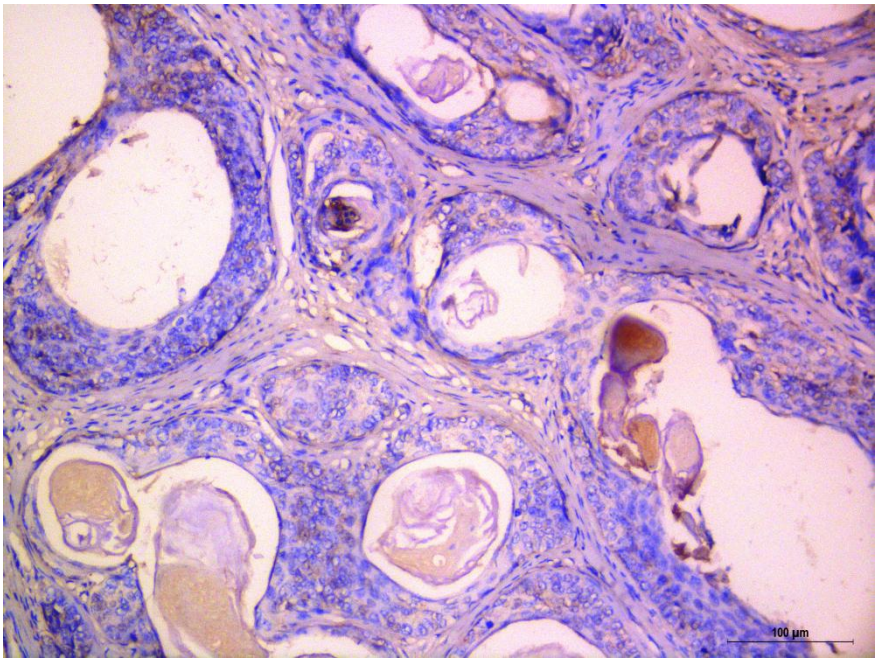

Plate 57. Trichoepithelioma. DEPTOR weak expression (IHC x200)

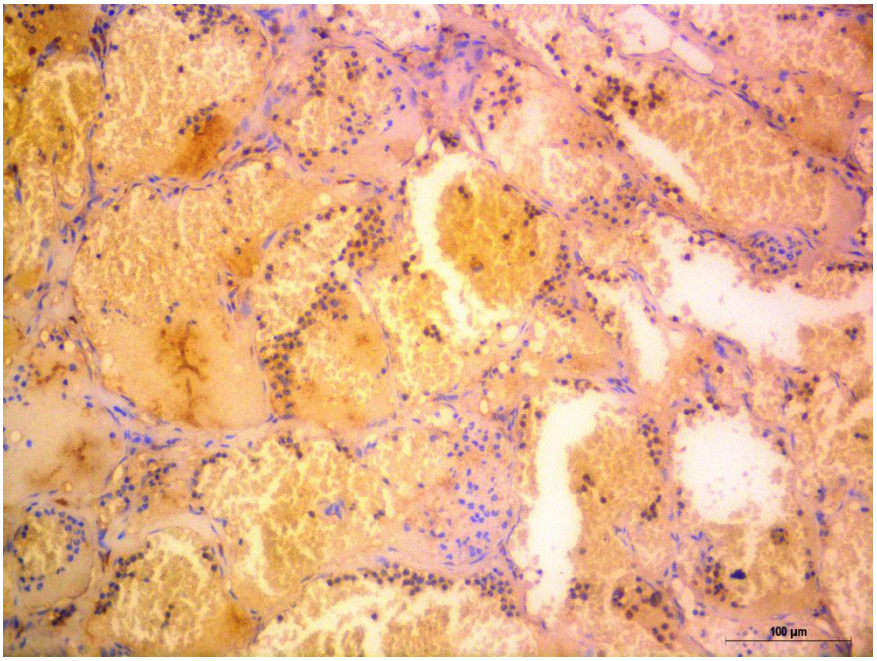

Plate 58. Haemangioma. DEPTOR weak expression (IHC x200)

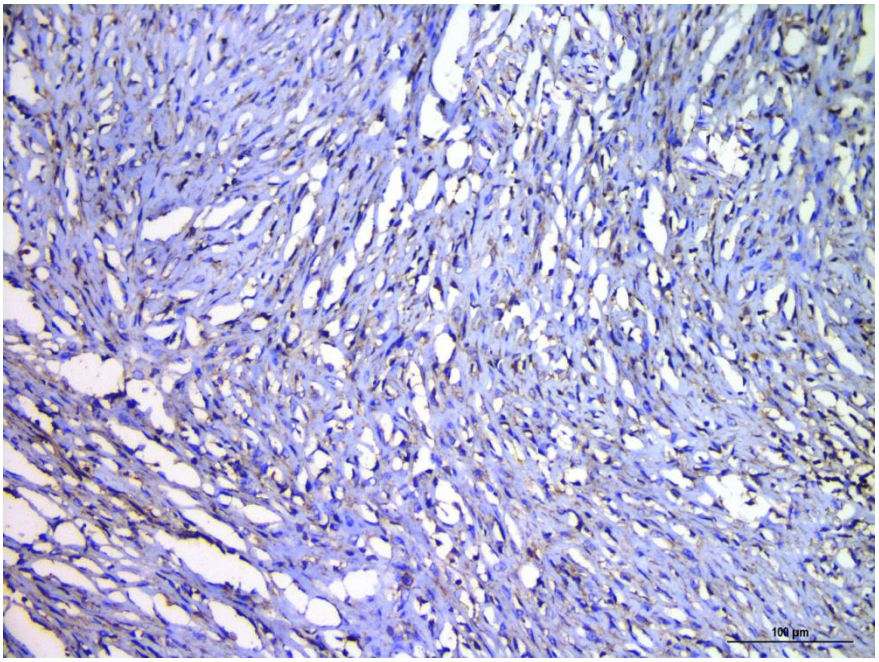

Plate 59. Fibrosarcoma. DEPTOR weak expression (IHC x200)

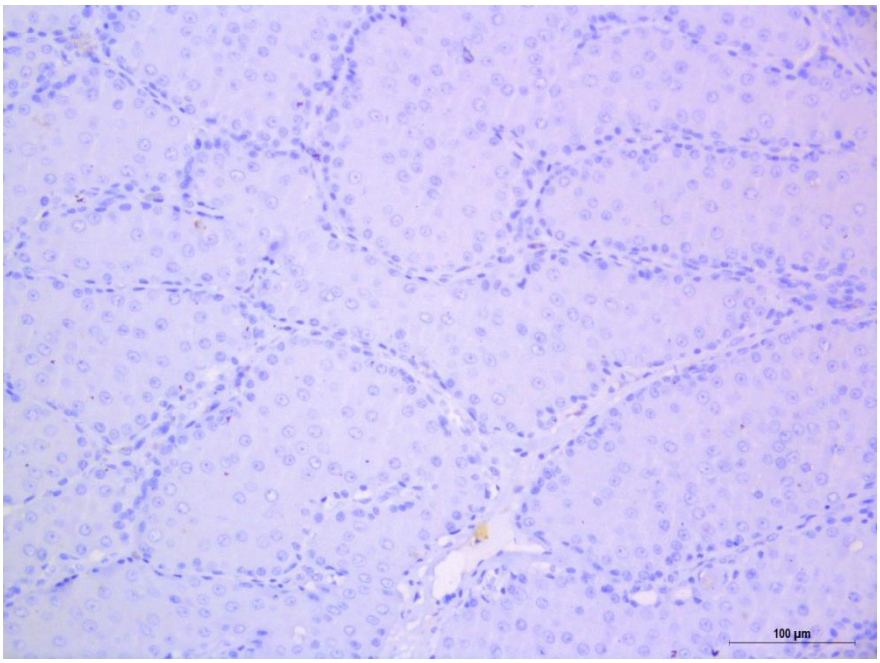

Plate 60. Hepatoid gland adenoma. DEPTOR weak expression (IHC x200)