The Investigation of Some Microenvironmental Markers in Canine Mast Cell Tumors

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Ph.D. Thesis

Supervisor: Prof. Dr. Hikmet KELEŞ

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INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF PATHOLOGY

Ph.D. THESIS

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16/11/2022

Muhammad Nasir BHAYA

ÖZET

Köpek Mast Hücre Tümörlerinde Bazı Mikroçevresel Belirteçlerin Araştırılması

Mast hücreleri, dokularda bulunan koruyucu-nöbetçi hücrelerdir. Mast hücrelerinin erken farklılaşması hematopoietik kök hücrelerden gerçekleşirken alternatif ve geç farklılaşması monosit granülosit öncülerinden gerçekleşir. Mast hücreleri birçok farklı tümörde kan damarlarının çevresinde ve ayrıca tümörlerin kenarlarında bulunur. Mast hücreleri, tümör mikroçevresinde proinflamatuar ve antitümörojenik rol oynar. Aktivasyon ve degranülasyondan sonra, antitümöral rolü yönetmek için nötrofilleri, eozinofilleri, makrofajları ve edinilmiş bağışıklık sisteminin hücrelerini etkilerler. Ancak, mast hücreleri tümörün vaskülarizasyonunu ve invazivliğini kolaylaştıran anjiyojenik bileşikleri de serbest bırakır. Ayrıca, ekstraselüler matriksi bozan ve tümör hücrelerinin metastazına yardımcı olan matriks metallopepsidaz (MMP-9) üretirler.

Mast hücre tümörlerinin (MCT) kaynağı yine mast hücreleridir. Hayvanlarda, MCT'ler oldukça yaygın olarak köpeklerde, daha az yaygın olarak kedilerde bulunurken atl, sığır, keçi ve domuzlarda insanlardakine benzer şekilde nadiren bulunur. Köpek MCT'leri evcil köpeklerde en sık görülen tümörlerdir ve köpeklerdeki tüm deri tümörlerinin neredeyse %20'sini oluşturur. MCT'lerin yeri genellikle kutanöz, daha az yaygın olarak subkutanöz ve nadiren de ekstrakutanözdür.

Canlı hayvan kullanılmamasına rağmen, bu çalışmanın teyidi için Afyon Kocatepe Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'ndan AKUHADYEK-22-21 numaralı etik kurul onayı alındı. Bu çalışma için Patoloji Anabilim Dalı arşivinden 60 adet MCT numunesi seçildi. MCT numunelerinin 33’ü dişi ve 27’si erkek köpeklerden alınmıştı. Bu çalışmada 18 farklı köpek ırkı incelenmiştir. Çalışmada kullanılan köpeklerin 23’ü Golden Retriever, 5’i Dogo Argentinos, 3’ü Labrador, 3’ü Pug, 2’si French Bulldog, 2’si Siberian Huskies, 2’si Cane Corso, 2’si Jack Russel Terrier, 2’si Dachshund, 1’i Samoyed, 1’i German Shepherd, 1’i American Pitbull Terrier, 1’i Cocker Spaniel, 1’i English Pointer, 1’i

Terrier, 1'i American Bulldog, 1'i Boxer ve 8'i melezdi. Tümör örnekleri %10 tamponlu formalin solüsyonunda laboratuvara ulaştırıldı. Rutin histopatolojik laboratuvar işlemlerinin ardından dokular; dereceleme (grading), nekrozun değerlendirilmesi ve mitotik figürlerin sayımı (MC) yoluyla tanıya ulaşmak için hematoksilen ve eozin (HE) ile boyandı. Mikroçevresel belirteçlerin değerlendirilmesi için MCT'ler immunohistokimyasal yöntemle KIT, Ki67, VEGF, OPN, Oct-3/4 ve TNF-α belirteçleri ile boyandı. Belirteçler ile diğer histopatolojik değişkenler (MCT dereceleri, nekroz varlığı ve MC) arasındaki korelasyon da Ki-kare testi kullanılarak araştırıldı.

Köpek kutanöz MCT'lerinin gelişimi için köpeklerin ortalama yaşı 9.25 idi. Golden Retriever ve melez köpek ırkları yüksek oranda kutanöz MCT insidansı gösterdi. Tüm tümörler, kutanöz MCT'ler için Patnaik ve Kiupel derecelendirme sistemlerine göre teşhis edildi ve derecelendirildi. Patnaik sistemine göre 17 köpeğe 1'inci derece, 33 köpeğe 2'nci derece ve 10 köpeğe de 3'üncü derece MCT tanısı konuldu. Derecelerin yüzdeleri sırasıyla 1'inci derece (%28,33), 2'nci derece (%55) ve 3'üncü derece (%16,67) idi. Daha sonra Kiupel derecelendirme sistemine göre tümörlerin sınıflandırılması yapıldı. Tüm derece 3 tümörler yüksek dereceli tümörlerdi ve tüm derece 1 tümörler düşük dereceli tümörlerdi. Derece 2 MCT'lerden 25 tümör düşük dereceli, 8'i ise yüksek dereceli MCT olarak derecelendirildi. Kiupel derecelendirme sistemine göre, tümörlerin %70'i düşük dereceli, %30'u ise yüksek dereceli köpek kutanöz MCT'leri olarak derecelendirildi. Ardından, 3 derece ve 2 dereceli değerlendirme sisteminin karşılaştırılması yapıldı ve 2 dereceli derecelendirme sisteminin köpek kutanöz MCT'lerinin tanı ve prognozu için gözlemciler arası daha fazla tutarlılığa sahip olduğu bulundu. Köpek kutanöz MCT'leride temiz marjlarının yüzdesi %70 idi ve MCT'lerin %20'si kirli marjlar gösterdi. Diğer %10 MCT'lerdeki marjlar klinisyenler tarafından sağlanan eksik bilgiler nedeniyle net değildi. Tüm tümörlerin derinliği, kutanöz MCT'leri doğrulamak için kontrol edildi. Tümörler epidermis, yüzeyel dermis ve derin dermis olmak üzere üç alanda mevcuttu.

KIT antikoru ile yapılan boyamada, 32 (%53,33) MCT'de membranöz immunopozitiflik, 22 MCT'de (%36,67) granüler sitoplazmik immunopozitiflik ve 6 MCT'de (%10) yaygın

sitoplazmik immunopozitiflik saptandı. Bu çalışmada KIT immunopozitifliğinin diğer histopatolojik değişkenlerle ilişkisi Ki-kare testi ile araştırıldı ve KIT ile MCT dereceleri, mitotik sayı ve nekroz varlığı arasında anlamlı ($P<0.05$) korelasyonlar değerlendirildi. OPN, TNF-α, VEGF, Ki67 ve Oct-3/4 antikor ekspresyonlarının yoğunluğu nonspesifik pozitiflik (-), hafif pozitiflik (+), orta pozitiflik (++), yaygın pozitiflik (+++) olarak derecelendirildi. OPN ile 18 MCT (+), 17 MCT (++) ve 25 MCT (+++) immunopozitiflik gösterdi. TNF-α ile 33 MCT (+), 17 MCT (++) ve 10 MCT (+++) immunopozitiflik gösterdi. VEGF ile 26 MCT (+), 22 MCT (++) ve 12 MCT (+++) immunopozitiflik gösterdi. Ki67 ile 28 MCT (+), 16 MCT (++) ve 16 MCT (+++) immunopozitiflik gösterdi. Oct-3/4 ile 18 MCT (+), 16 MCT (++) ve 26 MCT (+++) immunopozitiflik gösterdi. Belirteçler (KIT, Ki67, VEGF, OPN, Oct-3/4 ve TNF-α) ile diğer histopatolojik değişkenler (nekroz ve MC varlığı) arasındaki korelasyon anlamlı kabul edildi. KIT ile diğer tüm belirteçler (Ki67, VEGF, OPN, Oct-3/4 ve TNF-α) arasında anlamlı bir korelasyon bulundu. Proliferasyon belirteçlerinin yüksek ifadesi ve KIT patern II ve III'ün ifadesi, köpeklerde daha az hayatta kalma süresinin göstergeleriydi. KIT patern II ve III ekspresyonunu gösteren MCT'lerde tirozin kinaz inhibitörleri tedavisi tercih edildi.

Marj değerlendirmesi, köpek kutanöz MCT'lerinin tanısında ve prognozunda önemli rol oynar. KIT, Ki67 ve VEGF, köpek kutanöz MCT'lerde farklılaşma, tanı, prognoz ve kemoterapi seçimi için daha önceki çalışmalarda tanımlandığı ve çalışmamızda doğrulandığı gibi güvenilir belirteçler olarak bulundu. Çalışmamızda immunhistokimyasal ve istatistiksel sonuçlar değerlendirildikten sonra ilk kez bu çalışmada kullandığımız OPN, Oct-3/4 ve TNF-α'nın köpek kutanöz MCT'lerinin prognozu, farklılaşması, tanısı ve değerlendirmesi için iyi mikroçevresel belirteçler olabileceği sonucuna varıldı.

Anahtar sözcükler: Immunohistokimya, Köpek kutanöz mast hücre tümörü, KIT, Ki67, Mast hücreleri, Mikro çevre, OPN; Oct-3/4, TNF-a, VEGF

SUMMARY

The Investigation of Some Microenvironmental Markers in Canine Mast Cell Tumors

Mast cells are sentinel cells reside in the tissues. Early differentiation of mast cell takes place from hematopoietic stem cells. The alternative and late differentiation of mast cells takes place from the monocyte granulocyte progenitor. Mast cells are found around blood vessels in many different tumors and also at the edges of tumors. Mast cells play proinflammatory and antitumorigenic role in the tumor microenvironment. After activation and degranulation, they recruit neutrophils, eosinophils and macrophages and the cells of acquired immune system to manage antitumoral role. However, mast cells also release angiogenic compounds that facilitate tumor vascularization and invasiveness. They also produce matrix metallopeptidase 9 (MMP-9) that degrade extracellular matrix and helps in the metastasis of tumor cells.

Mast cells are the origin of mast cell tumors (MCTs). In animals, MCTs are found quite commonly in dogs, less commonly in cats and are rarely found in horses, cattle, goats and pigs, similar to that in humans. Canine MCTs are the most common tumors in domestic dogs accounting for almost 20% of all skin tumors in dogs. Location of MCTs is commonly cutaneous, less commonly subcutaneous and rarely extracutaneous.

Although no live animals were included, ethics committee approval was obtained from Afyon Kocatepe University Animal Experiments Local Ethics Committee with the number AKUHADYEK-22-21 for the confirmation of this study. Sixty samples of MCTs from the archive of Department of Pathology were selected for this study. The MCT samples were taken from 33 female and 27 male dogs. Eighteen different dog breeds were presented in this study. Twenty-three Golden Retrievers, 5 Dogo Argentinos, 3 Labradors, 3 Pugs, 2 French Bulldogs, 2 Siberian Huskies, 2 Cane Corso, 2 Jack Russel Terrier, 2 Dachshund, 1 Samoyed, 1 German Shepherd, 1 American Pitbull Terrier, 1 Cocker Spaniel, English Pointer 1, Terrier 1, American Bulldog 1, Boxer 1 and 8 dogs of mix breeds were also included in this study. Tumor samples arrived at the laboratory in 10% buffered formalin solution. After the routine procedures of histopathological laboratory, the tissues were

stained with hematoxylin and eosin (HE) for the diagnosis via grading, evaluation of necrosis and counting of mitotic figures (MC). For the evaluation of microenvironmental markers, the MCTs were stained with C-kit, Ki67, VEGF, OPN, Oct-3/4, and TNF-α markers by immunohistochemical method. The correlation between markers and other histopathological variables (grades of MCTs, presence of necrosis and MC) was also investigated with the use of Chi-square test.

The mean age of dogs was 9.25 years for the development of canine cutaneous MCTs. The Golden Retriever and mixed breeds of dogs showed high incidence of cutaneous MCTs. All the tumors were diagnosed and graded according to the Patnaik and Kiupel grading systems for the cutaneous MCTs. According to the Patnaik system, 17 dogs were diagnosed as grade 1, 33 dogs as grade 2 and 10 dogs as grade 3 MCTs. The percentages of grades were as follows, grade 1 (28.33%), grade 2 (55%) and grade 3 (16.67%). After that, the classification of tumors was done according to the Kiupel grading system. All the grade 3 tumors were high grade tumors and all the grade 1 tumors were low grade tumors. Twenty-five tumors from the grade 2 MCTs were graded as low grade and 8 tumors were graded as high grade MCTs. According to the Kiupel grading system, 70% tumors were graded as low grade and 30% were graded as high-grade canine cutaneous MCTs. Then, the comparison of 3-tier and 2-tier grading system was done and it was found that 2-tier grading system has more inter-observer consistency for the diagnosis and prognostication of canine cutaneous MCTs. The percentage of clean margins of canine cutaneous MCTs was 70% and 20% MCTs showed dirty margins. Other 10% MCTs were not clear because of the incomplete information provided by the clinicians. The depth of all the tumors was checked to confirm the cutaneous MCTs. Tumors were present in three areas including epidermis, superficial dermis and deep dermis.

Thirty-two (53.33%) MCTs revealed membranous immunopositivity, 22 (36.67%) MCTs revealed stippled cytoplasmic immunopositivity and 6 (10%) MCTs revealed diffused cytoplasmic immunopositivity via KIT antibody. The relation of KIT immunopositivity with the other histopathological variables was investigated with Chi-square test and

significant ($P<0.05$) correlations were evaluated between KIT and grades of MCTs, mitotic count (MC) and the presence of necrosis in this study. Intensity of OPN, TNF-α, VEGF, Ki67, and Oct-3/4 antibody expressions was graded as nonspecific positivity (-), mild positivity (+), moderate positivity (++), diffuse positivity (+++). Eighteen MCTs showed (+), 17 MCTs showed (++) and 25 MCTs showed (+++) immunopositivity with OPN. Thirty-three MCTs showed (+), 17 MCTs showed (++) and 10 MCTs showed (+++) immunopositivity with TNF-α. Twenty-six MCTs showed (+), 22 MCTs showed (++) and 12 MCTs showed (+++) immunopositivity with VEGF. Twenty-eight MCTs showed (+), 16 MCTs showed (++) and 16 MCTs showed (+++) immunopositivity with Ki67. Eighteen MCTs showed (+), 16 MCTs showed (++) and 26 MCTs showed (+++) immunopositivity with Oct-3/4. The correlation between the markers (KIT, Ki67, VEGF, OPN, Oct-3/4 and TNF-α) and other histopathological variables (presence of necrosis and MC) was considered significant. A significant correlation was found between KIT and all other markers (Ki67, VEGF, OPN, Oct-3/4 and TNF-α). High expression of proliferation markers and expression of KIT pattern II and III were the indicators of less survival time in dogs. Tyrosine kinase inhibitors therapy was preferred in the MCTs showing expression of KIT pattern II and III.

Margin evaluation plays important role in the diagnosis and prognostication of canine cutaneous MCTs. KIT, Ki67, and VEGF were found to be reliable markers for differentiation, diagnosis, prognostication, and selection of chemotherapy in canine cutaneous MCTs, as identified in previous studies and confirmed in our study. In our study, after evaluating the immunohistochemical and statistical results, it was concluded that OPN, Oct-3/4 and TNF-α, which we used for the first time in this study, may be good microenvironmental markers for the differentiation, diagnosis and prognostication of canine cutaneous MCTs.

Key words: Canine cutaneous mast cell tumor, KIT; Ki67, Mast cells, Microenvironment, Immunohistochemistry, OPN, Oct-3/4, TNF-α, VEGF

PREFACE

This PhD thesis assessed the evaluation of micro environmental markers in canine cutaneous MCTs. Micro environmental markers (KIT, Ki67, VEGF, OPN, Oct-3/4, and TNF-α) were proposed to have an essential role in the distinction, diagnosis, margin evaluation, and prognosis of canine cutaneous MCTs. These four years have been a difficult journey, with both ups and downs. Living away from family in a foreign country was a difficult time. On my journey, I was not alone, but was joined by a large team of specialists. Prof. Dr. Hikmet KELEŞ was my great supervisor throughout these years: "You are full of information and ideas and were always willing to find time for me" You are a wonderful person and my favorite. Thank you for always being there for me. In addition, I would like to thank my second mentor, Assist. Prof. Dr. Mehmet Fatih BOZKURT, for his comments and ideas on my work, as well as his continuous financial assistance. Thank you for exposing me to the laboratory and practical work. I would like to thank Afyon Kocatepe University Scientific Research Projects Coordination Unit (BAPK) for the funding of this thesis project (21.SAG.BIL.13).

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Muhammad Nasir BHAYA
Afyonkarahisar
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CONTENTS

ABBREVIATIONS

°C = Degree centigrade

5-HT1A = 5-hydroxy tryptamine

Cm = Centimeter

Cox-2 = Cyclooxygenase 2

DFI = Disease free interval

DNA = Deoxyribonucleic acid

ELIZA = Enzyme-linked immunosorbent assay

HE = Hematoxylin and eosin

HPF = High power field

IL = Interleukines

ITD = Internal tandem duplication

KIT = Proto oncogene c-kit

MC = Mitotic count

MCM7 = Minichromosome maintenance complex component

MCTs = Mast cell tumors

MDM2 = Mouse double minute 2 homolog

mg/kg = Milligram per kilogram

mm = Millimeter

MMP-9 = Matrix mettalopeptidase -9

MST = Median survival time

NKcs = Natural killer cells

Oct-3/4 = Octamer binding transcription factor 3/4

OPN = Osteopontin

PCR = Polymerase chain reaction

SPP1 = Secreted phosphoprotein

TNF-α = Tumor necrsis factor alpha

VEGF = Vascular endothelial growth factor

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1. INTRODUCTION

1.1. General Information about Mast Cells

Mast cells are sentinel cells reside in the tissues. Early differentiation of mast cell takes place from hematopoietic stem cells (Chen et al., 2005). The alternative and late differentiation of mast cells takes place from the monocyte granulocyte progenitor (Arinobu et al., 2009). Mucosal mast cells and connective tissue mast cells are the two different types that are identified in rodents. This classification is on the basis of protease contents, tissue localization and morphological characteristics. Mucosal mast cells show expression of chymase and connective tissue mast cells show expression of tryptase. The other difference in these mast cells is the expression of heparin in the secretory granules and connective tissue mast cells show higher expression of it (Enerbäck, 1966; Befus et al., 1982; Berman and Ross, 1984). Depending on the expression of chymases, tryptases and different proteases in the secretory granules, human mast cells are also divided into two types (Irani et al., 1986). Mast cells that contain only tryptase, localize with the T cells in the mucosa of lungs and intestines. Other mast cells that contain tryptase, chymase and other proteases reside in the connective tissues, skin, breast parenchymal tissues, lymph nodes, conjunctiva, synovium and the submucosa of gastrointestinal tract (Khazaie et al., 2011). Cutaneous mast cells of dogs revealed similarities with the human mast cells having tryptase (Meuten, 2017). Mature mast cells rarely present outside of the connective tissues in mice. Isolated mast cells were identified in the crypt region along with the epithelial stem cells. Mast cells are also present in the hair follicles along with stem cells and involved in the maturation and growth process of hair (Arck et al., 2001). Mast cells normally show migration and reside in the tissues as progenitors (Hallgren and Gurish, 2007). Hair follicles and the intestinal mucosa are the main source of mast cell progenitors (Kumamoto et al., 2003; Gounaris et al., 2007). Lymph nodes, bone marrow, spleen, peripheral blood and gut mucosa are the places where undifferentiated mast cell progenitors reside. The differentiation process of these progenitors into chymase expressing mature mast cells takes

place according to the requirement of tissues (Kasugai et al., 1995; Rodewald et al., 1996; Chen et al., 2005).

1.2. Role of Mast Cells in the Tumor Microenvironment

A variety of cells such as fibroblasts, blood vessels, extracellular matrix, signaling molecules and tumor cells are the main components to shape the microenvironment of tumor (Hui and Chen, 2015). The tumor microenvironment can be the site of inflammation where the mediation of inflammatory response takes place by the release of cytokines, chemokines, and different other enzymes by the infiltrated and resident cells. These releasing factors are VEGF, iNOS, IL-6, TNF-α, MMP-9 and Cox-2 (Huang et al., 2008). Mast cells reside around the blood vessels in the tumors and also at the margins of tumors (Tamma et al., 2017). The presence of mast cells in the tumors has been identified by the first time in 1878 by Paul Ehrlich (Domenico Ribatti and Crivellato, 2012). Mast cells have controversial effects and play proinflammatory and antitumorigenic role in the tumor microenvironment. After activation and degranulation, they recruit neutrophils, eosinophils, and macrophages and the cells of acquired immune system to manage antitumoral role (Hempel et al., 2017). Mast cells also release VEGF to support angiogenesis for the progression of tumors. They also produce MMP-9 that degrade extracellular matrix and helps in the metastasis of tumor cells (Hempel et al., 2017). The presence and role of mast cells has also been studied in animal models (Popivanova et al., 2008; Fu et al., 2017). Somatic cell factors and chemokine ligands are the main chemoattractants that help in the accumulation of mast cells in the tumor microenvironment (Yu et al., 2018). Mast cells release angiogenic compounds such as VEGF, FGF-2, IL-8, MMP-9 and MMP-2 and those compounds facilitate vascularization and invasiveness of tumors (Ribatti and Crivellato, 2012). Mast cells also release cytokines such as IL-1, MCP-3, IL-8, IL-4, TNF-α and also chymase and those contribute in the development of inflammation and inhibiting the growth of tumor cells (Ribatti and Crivellato, 2012). Immunosuppressive role of mast cells has also been identified by releasing histamine, TNF-α, and IL-10 in tumors. Additionally,

mast cells can also suppress T cells and NK cells in the tumor microenvironment by release of adenosine (Huang et al., 2008).

1.3. Mast Cell Tumors

MCTs are found commonly in dogs (Hottendorf and Nielsen, 1967; London and Seguin, 2003) less commonly in cats (Blackwood et al., 2012; Henry and Herrera, 2013), and rarely in humans (Furitsu et al., 1993; Longley et al., 1996), horses (Millward et al., 2010), cattle (Smith and Phillips, 2001), goats (Khan et al., 1995) and, pigs (Bean-Knudsen et al., 1989; Martínez et al., 2011). Mostly, MCTs are present in adult animals but interesting and rare cases called as mastocytosis has also been reported in puppies (Davis et al., 1992a), foals (Cheville et al., 1972) calves, (Smith and Phillips, 2001) and goat (Khan et al., 1995). These rare cases have resemblance with urticaria pigmentosa in humans (Furitsu et al., 1993; Longley et al., 1996). MCTs can be diagnosed from cytological examination of fine needle aspirates and hematoxylin-eosin-stained histopathological tissue sections. Although, cutaneous MCTs in dogs, cats and horses have been cured by proper surgical removal but a portion of canine cutaneous MCTs (Baginski et al., 2014; Krick et al., 2017) and a few cases in cats (Dank et al., 2002) and horses (Reppas and Canfield, 1996) have shown metastasis to the local lymph nodes. A great effort has been already made for characterization of histopathological and molecular features of canine MCTs that show more aggressive behavior.

1.4. Canine Mast Cell Tumors

Canine MCTs are the most common tumors in domestic dogs accounting for almost 20% of all skin tumors in dogs (London and Seguin, 2003; Blackwood et al., 2012; Davide Berlato et al., 2021). The location of MCTs is commonly cutaneous, less commonly subcutaneous and rarely extracutaneous, and with the increase in grade, the less aggressive behavior of the tumor changes towards a highly aggressive or metastatic form (Blackwood et al., 2012). Cutaneous MCTs in dogs are more aggressive than subcutaneous MCTs, and the assessment methods to be used vary. Mast cell leukemia or extracutaneous MCTs are rare

and present mostly in the intestinal tract in dogs (Endicott et al., 2007; Marconato et al., 2008). Canine cutaneous MCTs are the most frequently diagnosed skin tumors, representing almost 21% of all skin tumors. There is no specific age or sex predilection in dogs for developing cutaneous MCTs, but according to previous reported studies, the mean ages were 9 years (Hottendorf and Nielsen, 1967), 8.5 years (Hottendorf and Nielsen, 1967; Strefezzi et al., 2003), and 7 years (Sabattini et al., 2021) for the development of cutaneous MCTs in dogs. MCTs have been reported in 2-week-old dogs, and one dog was reported with multiple cutaneous mastocytic proliferation at 3 weeks of age, and the lesions spontaneously regressed over 28 weeks and were not visible by 35 weeks of age. The neoplastic nature of these lesions was not known, but they have similarities with urticaria pigmentosa syndrome in humans and have been referred to as mastocytosis (Davis et al., 1992a).

MCTs have been reported in many breeds of dogs but some breeds showed a greater number of tumors. The most commonly affected breeds are boxers, Labrador, golden retrievers, shar-peis, bulldogs, Boston terriers, pit bull terriers, fox terriers, weimaraners, cocker spaniels, Rhodesian ridgebacks, dachshunds, Australian cattle dogs, beagles, schnauzers, and pugs (Hottendorf and Nielsen, 1967; Miller, 1995; McNiel et al., 2006; White et al., 2011). MCTs have been reported to show less aggressive behavior in boxer and pug breed, but more aggressive behavior shar-peis breeds dogs (Miller, 1995; McNiel et al., 2006). This more aggressive behavior of cutaneous MCTs have significant association with the cutaneous mucinosis in Shar-peis has also been reported (Miller, 1995).

The most common sites of canine MCTs are cutaneous followed by subcutaneous sites but can also develop anywhere on the body of dogs. Boxers, pugs, Boston and Staffordshire terriers showed high incidence of MCTs on the hind legs but Rhodesian ridgebacks more commonly developed MCTs on the tail, and English setters on the head and hind legs (Hottendorf and Nielsen, 1967; Bostock, 1973; McNiel et al., 2006; White et al., 2011; O'Connell and Thomson, 2013). Extracutaneous MCTs may be found in the

gastrointestinal tract, oral cavity, salivary glands, conjunctiva, nasopharynx, larynx, lungs, liver, spleen, urethra, and spinal cord. On the other hand, cutaneous MCTs may also develop metastases in different organs (Hottendorf and Nielsen, 1968; O'Keefe et al., 1987). Mast cell leukemia or disseminated MCTs are almost rare in dogs. MCTs mostly develop as a solitary nodule like lesion but multiple skin masses of cutaneous MCTs have also been reported in different breeds of dogs such as boxers, Boston terriers, golden retrievers and pugs (Bostock, 1973; Mullins et al., 2006; O'Connell and Thomson, 2013). Multiple MCTs ranging from 2 to 7 have been reported in pugs in one study at different anatomic locations and they found 56% of pug dogs with multiple simultaneous MCTs (McNiel et al., 2006).

MCTs are linked to the release of heparin, histamine and proteases and they have a significant relationship with local and systemic paraneoplastic signs in dogs. MCTs may develop erythema, ulcers, and Darier's sign due to the degranulation of neoplastic mast cells. Histamine H1 receptors cause erythema, local swelling and pruritus, but histamine H2 causes gastrointestinal ulceration in canine MCTs. Hydrochloric acid over secretion and hyper-motility have been mediated through histamine H2 receptors and anorexia, abdominal pain, vomiting, gastrointestinal hemorrhages and ulceration are the main consequences due to stimulation of gastric H2 receptors by MCTs in dogs. Peritonitis has been reported in some animals due to the perforation of gastric ulcers and the main cause is gastric H2 receptors. Gastrointestinal hemorrhages cause bleeding and bleeding causes iron deficiency that leads to secondary anemia in dogs. Aggressive large MCTs or widespread tumors cause massive release of histamine in dogs and which may cause collapse or anaphylactic reaction in dogs (Meuten, 2017).

1.5. Gross Morphology

The gross appearance of cutaneous, subcutaneous and extracutaneous MCTs varies depending on tumor grading or aggressiveness and location. Cutaneous MCTs range from nodular rashes to diffuse swellings or hairless, raised, erythematous, or highly variable

tumors. These tumors also have different sizes ranging from a few millimeters to large tumors (Meuten, 2017). Solitary lesions are mostly well circumscribed, show a slow growing pattern and are often present for months. Aggressive tumors are mostly poorly circumscribed, have ulcerated and pruritic surfaces, and show rapidly growing behavior. Large, invasive and severely ulcerated MCTs show aggressive behavior like malignant tumors; however, well-differentiated MCTs should not be assumed to be benign because they can also show aggressive behavior. Cutaneous MCTs do not have distinct margins and their cut surface show white or pink, sometimes with foci of hemorrhages (Newman et al., 2007; Thompson, Pearl, et al., 2011). Sometimes, cutaneous MCTs are not well circumscribed and margin evaluation can be difficult on palpation or visualization during surgical excision. Metastatic spread of MCTs is evidenced by lymphadenopathy or organomegaly by palpation or imaging (Ferrari et al., 2020). Subcutaneous MCTs may develop anywhere on the body in the subcutis but the most common sites accounting for almost 60% of cases are the legs, thorax, and back (Newman et al., 2007; Thompson, Pearl, et al., 2011). Subcutaneous MCTs have been reported mostly as a single mass even in one study 95% were single masses and only 5% were multiple masses. They do not enter the dermis layer and are rarely ulcerated but they cause the skin to bulge. They grow like a soft fleshy mass that may be misdiagnosed as lipomas by gross examination (Thompson et al., 2011). MCTs may develop in the internal organs as a result of primary MCTs metastasis. Oral MCTs are very uncommon in dogs, accounting for only 1.8% of the possibility of their development. MCTs showed involvement in the lips, buccal mucosa, tongue and gingiva of dogs (Vos and van der Gaag, 1987). Intestinal MCTs are more frequent in cats but some cases are also reported in dogs (Alroy et al., 1975; Iwata et al., 2000; Rissetto et al., 2011).

1.6. Histopathological Characteristics

Adipose tissues surround the subcutaneous MCTs in the subcutis especially in dogs but some tumors cells may extend into the deeper dermis and most tumors are subjacent to the dermis and epidermis. Tumors located in the epidermis or outer dermis are the cutaneous MCTs and the majority of tumors below this location are the subcutaneous MCTs. It shows

that prognosis of tumors also depends on location of tumors. Some subcutaneous MCTs were diagnosed as grade 2 cutaneous MCTs due to their deeper location in the dermis (Meuten, 2017). In one study (Thompson et al., 2011), it has been reported that the majority of subcutaneous MCTs were infiltrative (n = 163), some were well circumscribed (n = 50) and others were a combination of both (n = 90), and they were not encapsulated. Lower magnification has been used to recognize the distinctive histologic patterns of cutaneous and subcutaneous MCTs. Rows of ribbons formed by neoplastic cells have been reported in some cases of MCTs. Some tumors may develop edema and hemorrhages that may cause the formation of blue foci at the location of MCTs. In some tumors, numerous eosinophils are important for the diagnosis of MCTs and they can be observed easily at first observation. The neoplastic cells of cutaneous MCTs are identical with the subcutaneous MCTs and it is difficult to differentiate on that level (Thompson et al., 2011).

The differentiation of cutaneous from subcutaneous MCTs is based on gross and sub gross evaluations. Neoplastic cells may be individualized and have distinct borders, or these cells may be packed so closely that their borders are indistinguishable. Round to polygonal cells with round central to slightly eccentric nuclei has been observed on higher magnification. The cytoplasm of tumor cells shows a moderate amount, pale pink and contains granules that stain gray or blue with histopathological stain (hematoxylin and eosin) but purple with metachromatic stains (Toluidine blue, Giemsa) (Jose and Schoning, 1994).

Eosinophils are always present in canine MCTs and sometimes they are prominent type of cells in MCTs. During the evaluation of the margins of tumors aggregates of eosinophils beyond the border of the tumors have been observed but they should not be interpreted as part of the tumor. Collagenolysis, sclerosis, edema, necrosis and lymphocytic infiltration due to secondary inflammation are often seen in canine MCTs (Meuten, 2017). The assessment of surgical margins can be difficult due to these severe secondary lesions because they can mask the neoplastic cells in canine MCTs. Margins can easily be defined in well differentiated tumors because they are well delineated. Less differentiated tumors are very complex tumors and their margin evaluation is very difficult because they are infiltrative (Séguin et al., 2001).

The diagnosis of canine MCTs can be easy with cytological and histopathological examination but it can be difficult in some tumors. Determination of margins and grading of tumors are the real challenge in canine MCTs and histopathological or molecular tools are mostly required. MCTs have granules in the cytoplasm and in some examples the cytoplasmic granules are so numerous and densely stained that they obscure the nuclei, while in others the granules are inconspicuous and must be searched for at high magnifications. Heavily granulated tumor cells show scattered granules in the background and they can be easily diagnosed by histopathological examination (Meuten, 2017).

1.7. Cytological Features of Canine MCTs

Cutaneous MCTs are commonly detected via cytology, and the cells are distinguished by separate, individualized, tiny to medium-sized round cells with cytoplasmic metachromatic granules. However, detecting nuclei and cytological characteristics for grading may be difficult in these tumors, and these granules may impede MC estimation (Meuten, 2017). Wright's stain is primarily utilized for cytological evaluation of canine MCTs. Cytoplasmic granules in poorly differentiated MCTs may not be seen by histological inspection, even when stained with specific stains such as Giemsa, toluidine blue, or other histochemical stains (acid fast, Luna's, etc.), but they can be plainly seen in cytological preparations. Mast cell granules saturate the cytoplasm and are extracellular in this MCT when stained with Wright's dye. Wright's or Diff-Quik stains the cytoplasm of properly differentiated MCTs. However, the granules in certain MCTs will not stain consistently with Diff-Quik or any aqueous stain. Most Romanowsky stains, such as Wright's or Wright-Giemsa, are methanolic in nature and can stain mast cells and basophil granules. Aqueous stains, such as the commonly used "dip" stains, may not stain the cytoplasmic granules of mast cells, basophils, or big granular lymphocytes (Allison and Velguth, 2010). With Wright's stain, some granules can be seen in poorly-differentiated MCTs (Jose and Schoning, 1994).

If a suspected MCT does not exhibit obvious granules when stained with Diff-Quik, consider staining more slides with a methanol-based Romanowsky stain like Wright's stain. Methanolic stains, rather than aqueous stains, improve the visualization of cytoplasmic

granules in canine MCTs. In canine MCTs, central nuclei surrounded by copious cytoplasm resemble a "fried egg." Eosinophil aggregates are typically detected in canine MCTs, and a round cell tumor with eosinophils admixed can also be classified as an MCT. Spindle cells are normally found in small quantities in highly cellular samples. These are stromal cells that provide support (Meuten, 2017). Researchers evaluated cytological and histological characteristics in one study (Scarpa et al., 2016), utilizing the 2-tiered histologic grading method as the gold standard. In that study, histologic grade was predicted accurately in 94% of cytology instances.

However, the study did not look into patient survival and instead relied on histologic criteria rather than developing a new cytological grading system (Scarpa et al., 2016). In another study (Camus et al., 2016), three board-certified clinical pathologists assessed cytological materials blindly. Cell granularity, nuclear pleomorphism, collagen fibrils, mitotic patterns, binucleation or multinucleation, and anisokaryosis were all assessed on a single properly cellular cytology slide. Granularity was classified as well granulated, badly granulated, or mixed (a mix of poorly granulated and well granulated cells). Nuclear pleomorphism was classified as present when non-rounded nuclear forms were seen, and as missing when only round to ovoid nuclear shapes were observed. It was determined whether collagen fibers, mitotic figures, and binucleated or multinucleated cells were present.

Anisokaryosis was characterized as a nuclear size variation of greater than 50%. The goal of that multi-institutional prospective study was to use the 2-tier grading standards as a guide to produce an accurate and repeatable cytological grading system for MCTs that predicts patient outcome (Camus et al., 2016).

1.8. Histological Grading of Canine Cutaneous MCTs

The most often utilized prognostic and therapeutic factor for canine MCTs is MC. Bostock (Bostock, 1973) and Patnaik (Patnaik et al., 1984) described the most commonly used

histologic grading systems in 1973 and 1984, respectively, and classified MCTs into three grades: well differentiated tumors as grade 1, intermediately differentiated tumors as grade 2, and poorly differentiated tumors as grade 3. Tumor grades correlate with clinical outcomes in both MCT grading schemes. Later in 2011, a new grading system was implemented, which categorizes MCTs as low grade or high grade (Kiupel et al., 2011). This two-tier categorization predicts overall survival, MCT associated mortality, and latency to new tumor growth in canine MCTs with 97% inter-observer agreement (Kiupel et al., 2011). The presence of any of the following criteria is used to diagnose high-grade MCTs in this two-tier categorization system: (1) at least seven mitotic figures in ten high-power-field (HPF) images; (2) at least three multinucleated (3 or more nuclei) cells in ten HPF images; (3) at least three bizarre nuclei in ten HPF images; and (4) karyomegaly (nuclear diameters of at least 10% of neoplastic cells vary by at least two times) (Kiupel et al., 2011). The main fields for determining tumor grade are the highest degree of anisokaryosis and the highest degree of mitotic activity.

Each of these fields will not be found in low-grade MCTs and the margins can easily be identified in in low-grade MCTs because they are well circumscribed (Bostock, 1973; Patnaik et al., 1984). In one study (Kiupel et al., 2011), it has been reported that almost 90% of cutaneous MCTs are low-grade tumors. The median survival time (MST) for different grades of MCTs is different because in that study (Kiupel et al., 2011), it has been reported that low grade MCTs have approximately 2 years and less than 4 months for high grade MCTs. In that study, 10 dogs had high-grade tumors, and 9 of them died due to MCT-associated disease (Kiupel et al., 2011). Five dogs from this study showed metastases in internal organs, meaning they were extracutaneous MCTs. There have been 85 low-grade MCTs reported in this study, but only 4 of them died due to MCT-associated disease and the percentage of mortality was only 5%, but 14 (17%) dogs with low-grade MCTs developed additional MCTs. From the 10 dogs with high-grade MCTs, seven dogs showed additional MCTs in almost 6 weeks (Kiupel et al., 2011). In other studies researchers reported the prognostic usefulness and the higher inter-observer consistency of the two-tier

grading system of canine MCTs (Takeuchi et al., 2013; Vascellari et al., 2013; Stefanello et al., 2015; Sabattini et al., 2015).

In one study, total 53 dogs were used and 46 low-grade and 7 high-grade MCTs were identified. Researchers in that study also evaluated the mortality rate for both of these grades in dogs by taking the follow up data of 12 months. In that study, the mortality rate was 6% for low grade MCTs and 71% for high grade MCTs (n=5) (Vascellari et al., 2013). That study also reported grade 2 MCTs in 22 (70%) dogs, and 17 dogs from 22 showed survival of more than 12 months. It means 5 dogs with grade 2 MCTs have died due to MCT-associated disease (Vascellari et al., 2013). The same results were found in other study (Takeuchi et al., 2013), and they also confirmed the higher inter-observer consistency for the two-tier grading system in canine MCTs as compared to Patnaik three-tier grading system. In that study, they evaluated disease-free interval (DFI) and survival time for both two-tier and three-tier grading systems of MCTs and they found 7 dogs with grade 3 MCTs had significantly reduced survival time and DFI compared to 40 dogs with either grade 1 or 2 MCTs. In this study they reported that there was no significant difference in survival time between grade 1 and 2 MCTs (Takeuchi et al., 2013). In that same study, when a two-tier grading system was used, it was found that 19 dogs had high-grade MCTs and 28 dogs with low-grade MCTs and high-grade MCTs had shorter survival time and DFI as compared to low-grade MCTs (Takeuchi et al., 2013).

In a third research (Sabattini et al., 2015), Patnaik classified 18 MCTs (13.1%) as grade 1, 83 (61%) as grade 2, and 36 (26%) as grade 3, with percentage findings of 13.1% for grade 1, 61% for grade 2, and 26% for grade 3 MCTs (Sabattini et al., 2015). In this investigation, there was no significant difference in prognosis between grade 1 and 2 MCTs, while grade 3 MCTs were shown to be related to poor prognosis (Sabattini et al., 2015). After 12 months of follow-up data, the survival probability for grade 1 MCTs was 100%, 87% for grade 2 MCTs, and 16% for grade 3 MCTs. According to the two-tier grading system, all grade 1 MCTs were low-grade and all grade 3 MCTs were high grade. Following a year of data collection, 71 grade 2 MCTs were low-grade and 12 were high-grade, with 86% being

low grade and 14% being high-grade MCTs, respectively. The survival probability percentages for low grade MCTs were 94% and 46% for high grade MCTs, respectively (Sabattini et al., 2015). According to a two-tier grading scheme, all grade 1 MCTs were appraised as low grade MCTs and all grade 3 MCTs were evaluated as high grade MCTs in one notable research (Stefanello et al., 2015). The overall number of tumors in that research was 386, with 52 being grade 1 MCTs, 43 being grade 3 MCTs, and 44 being grade 2 MCTs. According to the two-tier method, 243 (84%) of the 291 Patnaik grade 2 MCTs were classified as low-grade MCTs, while 48 (16%) were classified as high-grade MCTs (Stefanello et al., 2015).

According to the two-tier grading system, dogs with grade 3 MCTs were much more likely to have metastases than dogs with grade 1 or 2 MCTs, and dogs with high-grade MCTs were significantly more likely to have metastases than dogs with low-grade MCTs (Stefanello et al., 2015). Cytology and histology revealed that 50 dogs had metastases to local lymph nodes, whereas 16 dogs had distant metastases. However, according to the two-tier approach, 3 of 52 (6%) dogs with Patnaik grade 1 MCTs, 48 of 291 (16%) dogs with Patnaik grade 2 tumors, and 44 of 295 (15%) dogs with low grade tumors had metastases at the time of diagnosis, with 38 MCTs having only local lymph node metastases. The authors of that study found that, while grading systems were useful for prognosis, no scheme was totally accurate, and staging remained the greatest overall predictor of cutaneous MCTs in dogs (Stefanello et al., 2015). Patnaik's three-tier grading system is extensively utilized, and significant follow-up data and result assessments are associated with it. They classified well-differentiated MCTs as grade 1, intermediately differentiated MCTs as grade 2, and poorly-differentiated MCTs as grade 3 in this grading (Patnaik et al., 1984). Separated by collagen bundles, distinct, round, monomorphic neoplastic mast cells with a round nucleus, no nucleolus, and no or infrequent mitoses (2 in 10 HPF). In dogs, they are restricted to the superficial dermis and are usually seen in inter-follicular gaps. In grade 1 MCTs, edema and necrosis are nonexistent or limited (Patnaik et al., 1984). Grade 2 MCTs include pleomorphic neoplastic cells that are less basophilic than their normal counterpart, indented nuclei with a single nucleolus, and rare mitotic figures (0-2 per HPF). Grade 2 MCTs are

also more cellular than grade 1 MCTs. In dogs, grade 2 MCTs are seen in the dermis, either superficially or deeply, and may infiltrate the subcutis and subjacent skeletal muscle.

In grade 2 MCTs, diffuse edema and necrosis are prevalent (Patnaik et al., 1984). Grade 3 MCTs are more cellular and pleomorphic than grade 2 MCTs, with tightly packed cells, irregularly shaped nuclei, and numerous nucleoli. In grade 3 MCTs, multinucleate and strange cells are abundant, and mitotic figures (3-6 per HPF) are common (Patnaik et al., 1984). Bostock's categorization likewise employed three differentiation-based categories, but the structure of the grades was different. Bostock utilized a new criterion, the nuclei-to-cytoplasmic ratio, that Patnaik did not employ, but all other criteria were the same (Bostock, 1973).

The fundamental distinction between the Patnaik and Bostock grading systems is the impact of tumor depth. Tumor depth is a main criterion utilized in the Patnaik method to differentiate grade 1 and 2 MCTs; however, there is no tumor depth criterion in Bostock's grading system. A significant degree of inter-observer variance was noted while grading grade 1 and grade 2 MCTs due to the uneven inclusion of tumor depth in the histopathologic categorization of canine MCTs (Bostock, 1973; Patnaik et al., 1984). Many pathologists use just cellular features to grade MCTs according to Bostock's classification and do not include tumor depth as required by Patnaik's classification, yet they assigned grade 1 to well differentiated MCTs in one study (Kiupel et al., 2005). They thus concluded that tumor depth should not be used in the histologic grading of canine cutaneous MCTs.

Cutaneous MCTs must be separated from subcutaneous MCTs in order to get accurate findings. If the bulk of the MCT is in the subcutis and is surrounded by adipose tissue, it is a subcutaneous MCT, and the MC is crucial. Both Bostock and Patnaik utilized large numbers of dogs in their investigations and found strong correlations between histologic grade and patient prognosis (Bostock, 1973; Patnaik et al., 1984). This association has been supported by several articles on canine MCTs throughout the years. Both researchers included only dogs in which the tumor had been entirely excised, excision was the sole

therapy; and there was no sign of metastases during the initial diagnosis; they also included comprehensive follow up data. Patnaik analyzed over 4.1 years of data from 83 dogs to conclude that 93% of dogs with well-differentiated MCTs survived the research term (1500 days) (Patnaik et al., 1984). Bostock examined 2.5 years of data from 114 dogs to conclude that 77% of pups with well-differentiated MCTs lived more than 30 weeks. Bostock also determined that pups who lived for more than 30 weeks were apparently healed, as this group of canines lived for the whole 2.5-year follow-up period (Bostock, 1973).

In all investigations, dogs with poorly differentiated MCTs had a dismal prognosis, with just 6% and 13% surviving more than 1500 or 210 days. They also tracked the number of dogs who died as a result of MCT-related sickness and their survival times. Recurrence and metastases in individual dogs were also documented, but there was no summary of this data, and DFI was not reported in either study (Bostock, 1973; Patnaik et al., 1984). It was also found that if MCTs were present for 28 weeks or longer prior to surgical removal, the afflicted dogs had a fair prognosis (Bostock, 1973). Some dog breeds have a higher risk of MCTs, such as Boxers, who have a high percentage of highly defined tumors. The mean survival duration of MCTs in different breeds of dogs is similarly diverse, with Boxer dogs surviving longer than other kinds.

In other investigations, grade 2 MCTs were shown to be as high as 70%. The 3-tier grading scheme of MCTs revealed inter-observer variance. These variances resulted in the use of a two-tier grading system for cutaneous MCTs. Many earlier investigations have indicated a higher level of these variations. Multiple studies have found a substantial degree of inter-observer variance (Kiupel et al., 2011; Northrup et al., 2005). Ten pathologists from a single institution assessed sixty canine cutaneous MCTs. When each pathologist was permitted to grade using his or her own set of histologic criteria, there was only 50.3% agreement, and when the Patnaik grading method was applied, total agreement climbed to 62.1% (Northrup et al., 2005).

A third research (Kiupel et al., 2011), found only 63% concordance for grade 1 MCTs, 63% concordance for grade 2 MCTs, and 74% concordance for grade 3 MCTs when 95 canine cutaneous MCTs were histologically rated by 31 pathologists from 16 different institutions (Kiupel et al., 2011). Others have utilized MC to better predict survival in grade 2 MCTs since, according to Patnaik, the MC for MCTs can range from 0 to 20 per 10 HPF (Patnaik et al., 1984; Romansik et al., 2007). Dogs with grade 2 MCTs with MC greater than 5 had a median survival time of 5 months, whereas MCTs with MC less than 5 had a median survival time of more than 70 months (Romansik et al., 2007). Nineteen dogs with MC more than 5 had a median survival time of two months, while 80 dogs with MC less than five had a median survival period of 70 months. In dogs, a high MC was related to a shorter survival time (Romansik et al., 2007). That study indicated that MC could predict canine survival time independent of grade (Meuten et al., 2016).

1.9. Histological Grading of Canine Subcutaneous MCTs

Canine subcutaneous MCTs should be distinguished from cutaneous MCTs. The main factors for the confirmation of subcutaneous MCTs are the involvement of the dermis, epidermis and adipose tissues. Grades were mostly not used in the subcutaneous MCTs. It was stated in previous studies that almost 90% of subcutaneous MCTs might have a benign nature and that surgical removal was the main treatment for canine subcutaneous MCTs (Newman et al., 2007; Thompson et al., 2011). There were different criteria for the identification of aggressiveness in canine subcutaneous MCTs. These criteria are MC greater than 4, Ki67 score (more than 22), AgNOR and Ki67 combined score (more than 55), diffused expression of KIT and multinucleation in 10 HPF (Thompson et al., 2011; Thompson et al., 2011). The most important criteria was the evaluation of MC, Ki67 and AgNOR × Ki67 combined scores (Thompson et al., 2011). The high scores and expression of these parameters were related to a shorter survival time in dogs. A survival time of 1, 2 and 5 years was found according to the aggressiveness and expression of the diagnostic parameters (Thompson et al., 2011). There was very low recurrence rate of canine subcutaneous MCTs which were well circumscribed and having clean margins. There were

2% chances of recurrence in the canine subcutaneous MCTs showing complete margins. The recurrence rate was only 12% in the canine subcutaneous MCTs showing incomplete margins. There were 11% of dogs that showed development of a second subcutaneous MCT away from the first MCT (Thompson et al., 2011).

1.10. Examination of Margins of Canine MCTs

The margin evaluation is the most important step in the evaluation of prognosis. Normal mast cells are already present in different organs. This makes it very difficult to find neoplastic mast cells in the presence of normal mast cells. This normal presence of mast cells also confuses the margin evaluation of MCTs. The margin evaluation for the low-grade MCTs in dogs is comparatively easy. Low-grade MCTs do not have capsules but the margins of these tumors are well delineated and clear. In the high grade MCTs, there is presentation of neoplastic cells around the primary tumor. These neoplastic cells play an important role in the complications of margins evaluation of high grade MCTs. Cutaneous MCTs also show different findings, like a reactive halo. This halo, which is formed around the capillaries in the skin tissues, contains mast cells, stromal cells, infiltrating inflammatory cells and edema fluids. The diameter of this halo can reach several centimeters. Due to the thickness of this halo, margin evaluation can be very difficult in canine cutaneous MCTs. Mast cells are found as single, multiple and group in this halo. Under the influence of different chemokines in inflammatory processes, inflammatory mast cells can be found together with neoplastic mast cells in this halo, and it is really difficult to distinguish inflammatory mast cells from neoplastic mast cells in the presence of such mastocytosis. In human studies they used CD25 for the identification of mastocytosis but it was not effective in the case of canine MCTs. Different experiments (IHC techniques) were used for the identification of neoplastic mast cells in canines. The tumors in grade 1 were marked with CD25 and KIT. The healthy skin tissue was marked only with KIT (Meyer et al., 2012). In allergic dermatitis, inflammatory mast cells were found, and these inflammatory mast cells are masking the neoplastic mast cells. CD25 was found to be a good marker for the identification of neoplastic mast cells in grade 1 MCTs. But when they

tried this marker for the high grade MCTs, the results were different, and they concluded that CD25 marker use is compromised for the high grade MCTs (Meyer et al., 2012). According to the researchers (Meyer et al., 2012), CD25 marker was also found positive for inflammatory mast cells in dogs and they concluded that the CD25 marker is not a perfect marker for the margin evaluation of canine MCTs. There is still need of any reliable marker for the identification of neoplastic mast cells in the presence of inflammatory mast cells. It will be a great development in the process of margin evaluation if someone finds a reliable marker. It was assumed that the criteria for the identification of neoplastic mast cells that neoplastic cells always will be in group or cluster form and the inflammatory mast cells will be present in the grade 1 or differentiated MCTs. This assumption is easy to make but the reality is that it is difficult to differentiate between neoplastic and inflammatory mast cells. It is a good thing that MCTs do not show so much recurrence even when neoplastic mast cells are present near the margin.

Although MCTs have a low chance of recurrence, clean margins are the correct prediction for non-recurrence. Researchers and the specialist still believe that if tumor cells are present near the margins, the recurrence chances will be high. Margins evaluation should not be reported in histopathological report if there is not clarity of neoplastic cells in the margins. The surgeon should report the surroundings of tumor if he cut any surface or other tissue. If he cut any surface tissue during surgery, he should mention it in his report, and it will help in margin evaluation. A good surgeon will always use ink to stain the margins of tumors before surgery. After surgery, tumor should be sent for proper margin evaluation with a proper report of surgical removal. There is no uniformly proved method for the margin evaluation. Parallel and tangential methods for the sectioning of tumor are present. Clean margins are always the best predictor of non-recurrence in MCTs of grade 1 (Scarpa et al., 2012). The recurrence rate in the grade 2 MCTs after the proper surgical removal is approximately 5-11%. A time period of 2-24 months has been reported for the recurrence of grade 2 MCTs (Séguin et al., 2001; Weisse et al., 2002). Incomplete excision increased the recurrence rate in both grade 1 and grade 2 MCTs, and this rate was 6-30%. In the grade 1 MCTs they have reported that 80-90% of the tumors did not show recurrence (Abadie et

al., 1999; Murphy et al., 2004; Séguin et al., 2006; Brocks et al., 2008). In some studies, it has been reported that low-grade tumors did not show any recurrence even after incomplete removal.

It was determined by different markers (Séguin et al., 2006; J. Smith et al., 2017). Follow-up is critical for the confirmation of recurrence. Histopathological and cytological methods should be used for the correct diagnosis, not the palpation of the tumors and regional lymph nodes. The important time for this process is when the dogs reveal different tumors. It was really confusing that MCTs' development near the site of the first tumor was recurrence or metastasis. The markers for the identification of recurrence or metastasis are very low in number. Mostly, c-kit has been used to distinguish the tumors. The correct diagnosis of tumors is really important for this recognition. Skin is not common site for the metastasis of tumors. The diagnosis should be clear, and if it is metastasis, it should be reported. The correlation between the primary tumor and a new tumor at a different site should be evaluated with the disease free interval (DFI) and the survival time of dogs. For the tumors smaller than 4 cm or grade 1 tumors skin margin of 2 cm and one facial plane (Simpson et al., 2004; Fulcher et al., 2006) are enough.

A margin of 1 cm is enough for both grade 1 and grade 2 MCTs, reported in another study (Schultheiss et al., 2011). Four-mm-deep margins were also enough for the complete removal of MCTs (Schultheiss et al., 2011). Lateral margins and fascial planes are important parameters for the successful removal and control of tumors (Pratschke et al., 2013). Three cm of lateral margin and one fascial plane have been reported for the complete removal of high-grade tumors (Donnelly et al., 2015). The distance of histologic free margin was not reported in that study. They also reported that 40% of high grade MCTs revealed recurrence even after complete surgical removal (Donnelly et al. 2015). While performing surgery, it is very difficult to know the grade of the tumor which is why there are chances of 20% recurrence, 80% metastasis and death in case of high grade MCTs. It has been reported that dog with grade 3 MCT has a 35% chance of living for at least two years. In the dogs with grade 2 MCTs this chance increased to 89%. In the dogs

having grade 1 MCTs have chance of 100% to live for 2 years (Donnelly et al., 2015). A Correct and consistent method should be used for the margin evaluation of MCTs in dogs. According to the recommendation of previous researchers, the evaluation of margins should include tangential margins and radial sectioning to confirm the distance. Cleanliness will be assessed by tangential margins and the distance between the margins and tumor cells will be assessed by radial sections of MCTs. They reported numerical values for different margins like infiltrated (M1), close (M2), and clean (M3 and M4). The diameters for these margins were also evaluated and these were 1-2 mm for M2, 2-5 mm for M3, and more than 5 mm for M4. These methods have consistency and provide accurate information in the margin evaluation of canine MCTs (Meuten, 2017).

1.11. Staging of Canine MCTs

This is really important for the diagnostic process of MCTs in dogs. Researchers (Mullins et al., 2006) use different tools for the staging. Cytological, histopathological, and clinical criteria are the main tools for this process. Different stages of canine MCTs have been reported (Krick et al., 2009). Solitary tumor that involves the dermis area but not metastasizes to the lymph nodes, is considered in stage 1. The tumors of stage 2 involve not only the dermis but also metastasize to the lymph nodes. Multiple tumors involving the dermis area with or without metastasizes in lymph nodes are considered stage 3 tumors. MCTs that have spread to other organs and tissues are classified as stage 4 tumors. Different studies (Mullins et al., 2006; Krick et al., 2009) have been reported in which the staging of MCTs is different, especially stage 3. No difference was found in the dogs of stage 1 and stage 3 but the dogs of stage 2 revealed a bad prognosis compared to the dogs of stage 3 (Murphy et al., 2006).

Fifty-four dogs were used in one study, and they evaluated DFI of >5 years in dogs having stage 3 MCTs (Mullins et al., 2006). A high grade skin tumor revealed a bad prognosis, but a small MCT on the limb area revealed a better prognosis during a study of canine MCTs (O'Connell and Thomson, 2013). Hayes et al. (2007) proposed a staging system for WHO,

but the staging system revealed no association with the previous studies. The European Society of Oncologists and Cancer Society recommended the staging system, but the involvement of lymph nodes was considered very important. The involvement of lymph nodes revealed a worse prognosis as compared to a normal cutaneous tumor. It was considered that lymph node involvement has main role for the detection of prognosis and staging of canine MCTs. Survival time was recorded in a study of canine MCTs, and the dogs of stage 1 revealed 6.2 years but the dogs of stage 2 revealed 0.8 years.

The aggressive or undifferentiated MCTs revealed high chances of metastasis in the local lymph nodes of dogs (Krick et al., 2009). High grade MCTs can be spread to lymph nodes and for the confirmation of this spread, a cytological examination of the nearest lymph nodes was recommended. If there is a chance of metastasis in the lymph nodes, it should be cleared before the tumor surgically removed. If no tumor was found in the lymph nodes with cytological examination, then there would be no need for lymph node removal during the surgical process. If metastasis was detected in the lymph nodes by any method, the lymph nodes should be removed along with the cutaneous tumor and both should be evaluated with histopathological examination (Baginski et al., 2014). For the confirmation of tumor cells, special stains can be used. Giemsa, toluidine blue and c-kit are the main special stains for the detection of MCTs.

C-kit evaluation is an immunohistochemical process used for the detection of neoplastic cells because it is really difficult to differentiate between neoplastic and inflammatory mast cells. Mast cells in the form of single or pairs can normally be present in the lymph nodes. Clusters of mast cells in the lymph nodes are the main criteria for the confirmation of metastasis in the lymph nodes. The relation between the cytological diagnosis of MCTs and the survival time of dog has been reported in one study.

They determined that 2-3 aggregates of multiple mast cells are enough to diagnose the metastasis in the lymph nodes (Krick et al., 2009). A large number of cells, aggregate presence of mast cells and pleomorphic cells are evidence of metastasis. In another study

(Krick et al., 2017), histopathological examination was done after the cytological examination, and the results of 20% positivity for metastasis were determined in canine MCTs.

Histopathological classification of MCTs was done on the basis of infiltration of mast cells, distribution of mast cells, and disruption of lymph nodes (Weishaar et al., 2014). Different metastatic stages of lymph nodes were given different names, like the pre-metastatic stage (HN1), the initial metastatic stage (HN2), and the late metastatic stage (HN3). These stages were based on the presence and distribution of mast cells in the lymph nodes. Metastatic lymph node revealed more than three mast cells in sinuses and at least 4 HPF. Less than 3 mast cells in the sinuses were considered as non-metastatic lymph nodes (Weishaar et al., 2014). Shorter survival time was recorded in the dogs having HN3 and HN2, respectively. The survival time of dogs with HN1 was recorded 1824 days but for the HN3 and HN2 this time was 804 days. That study revealed that the dogs having metastasis in the lymph nodes have worse prognosis as compare to the dogs with only cutaneous MCTs (Weishaar et al., 2014). The effect of chemotherapy was also examined in these dogs, and no effect of adjuvant therapy was recorded on survival time. A combination of lympho-scintigraphy and intra-operative lympho-scintigraphy with blue stain was used for the mapping of sentinel lymph nodes in different studies for the metastatic cases. Nineteen dogs were checked for the mapping process and 8 dogs revealed sentinel lymph nodes for the metastasis of MCTs. These lymph nodes were not removed during surgery. Tumors and the nearest lymph nodes were removed (Worley, 2014). MC of these MCTs with nodal metastasis was recorded and it was ≤5.36. There was a total of 12 dogs and 7 dogs revealed this MC.

C-kit mutation play a key role in the MCTs, and there should be consideration of PCR for the confirmation of mutation in both cutaneous and subcutaneous MCTs. After the confirmation of mutation tyrosine kinase therapy can be predicted. For the biological significance of MCTs, quality research should be considered for the determination of the molecular phenotype of mast cells. Especially the mast cells present in the peripheral area of the tumor or in the lymph nodes. As previously stated, high-quality research will help in

the detection of clean margins and the metastasis of MCTs in lymph nodes. Staging is not required for all tumors because the proper examination of lymph nodes is enough for most low grade MCTs. Staging is required for the determination of special therapy or high grade MCTs.

For the confirmation of low- or high-grade MCTs, fine needle aspirates and ultrasound should be recommended. If high-grade MCT is confirmed, then staging should be considered for proper handling. If there has been finding of metastasis in the lymph nodes, then there should be a full staging of the tumor for consideration of the proper therapeutic method. Ultrasonography of different body regions and aspirates from different organs should be recommended for this purpose. Routine cytology of different organs that are appear normal with ultrasound was not considered a good procedure for staging. Mast cells were detected during cytological examination of different organs, but the organs were normal with ultrasound examination. In that study, they evaluated the short survival time in the dogs with mast cell proliferation in different organs detected with cytological examination (Finora et al., 2006; Stefanello et al., 2009). The buffy coat of dogs with MCTs were evaluated in one study. They concluded the buffy coat examination is not useful because they have found more mast cells in the inflammatory diseases compared to canine MCTs (McManus, 1999). Due to the difficulty of confirmation of metastasis researchers can face difficulties in assigning stage 4 to the MCTs. Lack of necropsy, follow-up data, and records of euthanasia are the main problems to find out the metastasis of MCTs in the internal organs. That is the reason they have very limited data on the actual metastasis in the internal organs. A total of 17 dogs were used in a study, and 4 dogs revealed metastasis in internal organs. The grades of these 4 tumors were evaluated, and it was found that 3 dogs had grade 2 MCTs and 1 dog has grade 3 MCT (Gerritsen et al., 1998). In different studies researchers have not found any difference in organs with ultrasound examination of metastatic diseases. It was concluded that ultrasound evaluation is mandatory for the detection of metastasis. The correlation between the imaging process and the cytological examination of different organs was recorded in different studies (Sato and Solano, 2004; Book et al., 2011).

1.12. Prognosis of MCTs in Dogs

Accurate treatment is very important for the MCTs. For this purpose, it is necessary to accurately diagnose the tumor. A proper diagnosis of the tumor will provide an accurate therapeutic method. The medication is also expansive. To save owners money, it is important to prognosticate the MCTs. Accurate prognostication will provide an accurate method for medication. Metastatic diseases due to MCTs are very rare, because MCTs do not show metastasis. Subcutaneous and low grade cutaneous MCTs can be identified histologically, and it has been reported that 10% of dogs will die with subcutaneous MCTs and 5% of dogs will die with low-grade cutaneous MCTs in different studies (Thompson et al., 2011; Kiupel et al., 2011). These were the efforts to identify the proper prognosis so that the appropriate medication could be used. In another study, 15% of dogs were reported to have regional lymph node metastasis (Stefanello et al., 2015). It also has been hypothesized that 20% of low-grade cutaneous MCTs will also show other cutaneous MCTs. This MCT can be a little bit away from the first tumor (Kiupel et al., 2011). It is very difficult to recognize whether this tumor is a metastasis or a new tumor. These tumors were like new tumors. MCTs can be appear as a single tumor or as multiple tumors. It was found that 10-20% of dogs revealed MCTs as multiple. It is also difficult to evaluate the prognosis of these tumors.

In some studies, it has been reported that multiple MCTs had a worse prognosis, but in other studies, good prognosis has been reported (Kiupel et al., 2005; O'Connell and Thomson, 2013). In that study, 280 dogs were used and 59 of them had multiple MCTs, with good prognosis of these tumors. After the 12-and-24 month followups, no difference was found in the prognosis of multiple and single MCTs. In the opposite study, 10 dogs with multiple MCTs revealed systemic mast cell disease. All the tumors were evaluated separately, and grading was done. Multiple tumors do not meet the criteria for grade 3 because it is very difficult to see the prognosis of these tumors. The occurrence of these extra MCTs is a real problem for the owners and oncologists because it is related to medication. The aggressive behavior of cutaneous MCTs in the perineal and inguinal areas

has not been confirmed in different studies of dogs (Kosanovich et al., 2004; Kiupel et al., 2005; Sfiligoi et al., 2005). Other studies have found a high rate of metastasis in MCTs with mucous membrane, especially from muzzles. This metastasis was found in the regional lymph nodes. The percentage of this metastasis was also reported, and it was 55-72% (Gieger et al., 2003; Hillman et al., 2010; Elliott et al., 2016).

In one study, dogs with MCTs in the mucocutaneous and perioral regions had the same survival time in a study (Hillman et al., 2010). In dogs, lymph node metastasis was thought to be the predictor of survival time. The dogs with metastasis in regional lymph nodes revealed a shorter survival time (Hillman et al., 2010). A cytological examination of local lymph nodes should be done for the prediction of prognosis. Histologic grading is not possible with only cytological examination, but if the MC is greater than 5, it can be important for the prediction of survival time (Hillman et al., 2010). In dogs with metastasis in regional lymph nodes, a four-year survival time has been reported. Obesity also plays an important role in the development of MCTs in dogs. Obese dogs revealed a high risk of MCT development (White et al., 2011). MCTs with different behaviors and aggressive behavior revealed different clinical signs. It includes a high growth rate, inflammation and invasion of local tissues, ulceration of the epidermis and enlargement of local lymph nodes. Paraneoplastic signs are also important in the MCTs in dogs (Ginn et al., 2000; Mullins et al., 2006; White et al., 2011). Bostock was the first person to report the first clinical parameter for the prognosis of MCTs. He proposed that a dog having a MCT of more than 28 weeks prior to excision can reveal a good prognosis (Bostock, 1973). Now there are many microscopic and molecular parameters for the prognosis of MCTs. At first, only the surgical method was a parameter, but now these other parameters are more accurate for the prognosis of MCTs. A good prognosis parameter will help for the proper medication of MCTs. Histological grading and other molecular parameters are more accurate for the correct prognosis and medication of MCTs. The prognostic value of parameter is also related to the statistical value of the population. If the population is high then there are always exceptions in which clinical, histopathological and molecular features may favor the tumors (Meuten, 2017).

1.12.1. Proliferation of Different Cells

The proliferation of different cells is an important factor in the assessment of diagnosis and prognosis. The cells reported in canine MCTs are Ki67, AgNOR, MC, and proliferating cell nuclear antigen (PCNA). These cells are important for the estimation of survival time, metastasis, and DFI in dogs. The Ki67 index was calculated and reported in 1999 (Abadie et al., 1999). The other cell counts (AgNOR, PCNA and MC) have also been reported in different studies of MCTs in dogs (Bostock et al., 1989; Simoes et al., 1994; Webster et al., 2007; Maglennon et al., 2008; Vascellari et al., 2013). MC is the main process that has been performed in routine cases. The differences in the counts of all these cells are on the basis of counting, total areas, regions, view size, microscopes, and also differences in staining methods. It should be done with care so that duplication is not present in the results, especially during the diagnostic process. These limitations should be taken into account, and the results of MC, Ki67 and Ki67 × AgNOR scores are important for the diagnosis of MCTs in dogs (Abadie et al., 1999; Simoes et al., 1994; Kravis et al., 1996; Sakai et al., 2002; Séguin et al., 2006; Scase et al., 2006; Romansik et al., 2007; Ozaki et al., 2007; Webster et al., 2007; Maglennon et al., 2008; Vascellari et al., 2013; Berlato et al., 2015). The proliferation and counting of MC, Ki67 and AgNOR × Ki67 scores were also routinely reported for the prognosis of canine MCTs (Séguin et al., 2006), (Smith et al., 2017; Thompson et al., 2011; Webster et al., 2007).

The process of PCNA counting is also a good method for the estimation of prognosis but the problem is that there is no correct cut-off value for PCNA count. Due to this reason it is not used in the process of prognosis estimation (Abadie et al., 1999; Scase et al., 2006; Webster et al., 2007). The cell cycle is important because all the proliferation markers give different information about it. The growth fraction is the number of cells in the cycle and the proliferation rate is the rate of cell cycle progression. The phase index indicates the phase of the cell cycle. These three components are very important for the proliferation of markers. These components determine cellular proliferation. Ki67 is a fraction marker, AgNOR is a marker of proliferation rate, and MC and PCNA are the markers of phase

index. These three categories of cells are important for the identification of phases of the cell cycle (Webster et al., 2007; Berlato et al., 2015).

The growth fraction and its speed are the important factors in determining of proliferation of cells, and no single marker can give accurate results. All these components are related to each other. If one component shows an increase in its rate, then the other component will also show an increase in its rate. Due to this reason, the evaluation of one marker is not enough, and it can show wrong results. It is preferred to evaluate all the markers together. For the determination of recurrence risk, the AgNOR × Ki67 combined score has to be measured for low-grade canine cutaneous MCTs (Séguin et al., 2006; Smith et al., 2017). Incomplete excision of MCTs is also the cause of reoccurrence in dogs. 11% of dogs revealed this, and Ki67 and AgNOR × Ki67 scores were also calculated. The Ki67 score was <23 and AgNOR × Ki67 combined score was <54. 5 dogs out of 46 revealed reoccurrence of low grade MCTs in that study (Smith et al., 2017). The combined score of AgNOR × Ki67 is also important for the identification of some subcutaneous and low grade MCTs (Thompson et al., 2011). These proliferation markers have also been reported to identify grade 2 MCTs, and it was reported that they can show very aggressive behavior (Abadie et al., 1999; Simoes et al., 1994; Scase et al., 2006; Ozaki et al., 2007; Maglennon et al., 2008). More than 54 combined scores of AgNOR × Ki67 revealed the risk of mortality and metastasis in both cutaneous and subcutaneous MCTs (Webster et al., 2007; Thompson et al., 2011).

The Ki67 score alone gives 5% less accurate results of MCT-associated mortality as compare to combined score of AgNOR × Ki67 (Maglennon et al., 2008). Fifty-six dogs were used in a study and 8 dogs revealed grade 1 MCTs. Forty-one dogs revealed grade 2 and 7 dogs revealed grade 3 of MCTs. Fifteen dogs from the grade 2 and 3 dogs from the grade 3 MCTs revealed more than 54 combined score of AgNOR × Ki67 (Webster et al., 2007). The dogs revealed combined score of AgNOR × Ki67 above 54 were dead before 12 months and other dogs showing this combined score less than 54 survived for 2 years (Webster et al., 2007). The combined score of AgNOR × Ki67 is really important for the

prognosis and therapeutic purpose especially in the canine MCTs. The dogs having combined score of AgNOR × Ki67 above 54 but do not have internal tandem duplication (ITD) mutations in the exon 11 revealed good response of prednisolone/vinblastine combination. Tyrosine kinase inhibitor treatment was not impressive in these dogs (Webster et al., 2007). K i67 and AgNOR both should be determined properly and referred to oncologists for review (Bostock et al., 1989; Abadie et al., 1999; Webster et al., 2007; Maglennon et al., 2008). Decreased survival time has been recorded in the dogs showing more than 93 Ki67 positive cells out of 1000 cells (Abadie et al., 1999). Twenty nine dogs of grade 2 showed Ki67 score less than 93 revealed survival time of 12 months but 10 dogs having Ki67 score more than 93 were died before 12 months (Abadie et al., 1999). Total 70 dogs were used in other study and 14 dogs having grade 2. Image analysis was done in that study, and MCTs revealed the percentage of neoplastic cells was 1.8%. These MCTs revealed positivity for Ki67 and median survival time was 395 days for those dogs the 1-, 2-, and 3-year probabilities of survival were 0.43, 0.21, and 0.21 (Maglennon et al., 2008). In another study, 70 dogs were used and 56 dogs revealed grade 2 MCTs. The percentage of neoplastic cells was less than 1.8%. Ki67 positivity revealed survival probabilities of 0.92, 0.86, and 0.77. The median survival time was not calculated in that study (Bostock et al., 1989).

Ocular grid was used in a study to avoid the inconsistency of different microscopes and it was stated that MCTs showing Ki67 positive cells more than 23 were worse. The progression of disease and MCT related mortalities are associated with this score of Ki67 (Webster et al., 2007). This methodology was also used in another study, and the cut-off value was 21.8. It was stated that this value is prediction of metastasis in the canine subcutaneous MCTs (Thompson et al., 2011). Ki67 and combined score of AgNOR × Ki67 were also evaluated in the subcutaneous MCTs of dogs. It was stated that the score of both of these parameters is highly associated with metastasis and recurrence (Thompson et al., 2011). In other study two-tier and three-tier grading system was used. In that study, Ki67 index was compared in the cutaneous MCTs (Vascellari et al., 2013). The Ki67 index value was 2.5, 6.0, and 20.6 according to the Patnaik grading system. These values were for all

three grades of MCTs respectively. Ki67 index was significant when compared with grade 1 MCTs and the combined grade 2 and 3. But this Ki67 index was less significant when compared with only grade 2 MCTs (Vascellari et al., 2013).

The first significant Ki67 index value was ($p = 0.024$) and the less significant Ki67 index value was ($p = 0.092$) (Vascellari et al., 2013). Grade 2 MCTs revealed so much diversity in the values of Ki67 index and it indicated that this group is important for the proper grading. The Ki67 index value was significantly lower in the low-grade MCTs as compared to high-grade MCTs. this value was ($p = 0.002$). It was stated that MCTs associated mortality is directly related to the high value of Ki67 index. This significant Ki67 index value was ($p = 0.049$). According to the established cut-off value, the sensitivity and specificity of grade 2 MCTs have been evaluated at different percentages (Vascellari et al., 2013).

These percentages were 62.5% and 82.2%, respectively on the basis of cut-off value. Total 40 rats were involved in this group and only 3 dogs died due to MCT related diseases. The Ki67 index was less than 10.6 in the case of those dogs. The survival probabilities were also evaluated and these were 0.95 and 0.92 for low-and high-grade MCTs, respectively (Vascellari et al., 2013). Five out of 13 dogs died due to MCT related diseases, and the Ki67 index was more than 10.6. The survival probabilities were also calculated and the values were 0.92 and 0.77. The survival time was 6 months and 12 moths, respectively for the low and high grade MCTs (Vascellari et al., 2013).

Significantly decreased survival time was evaluated in different studies. These studies were performed on the cutaneous MCTs in dogs. The mean AgNOR count was recoded and it was stated that AgNOR count higher than 2.25 or 4 AgNORs/cell may indicate MCT related diseases and the chances of the death of the dogs were higher (Bostock et al., 1989; Simoes et al., 1994). No mortality was reported in the dogs with AgNOR count less than 1.7. But the dogs with AgNOR count more than 4 were 12 out of 18 and they died due to MCT related diseases (Bostock et al., 1989). Some other researchers also tried to evaluate

the relation of AgNOR count and prognosis but they failed to establish a proper cut-off value for it (Scase et al., 2006; Webster et al., 2007). The process of MC was also considered important for the prognostication of MCTs in dogs. This process was considered important to evaluate the proliferation of cells in the tumor. MC is used alone or in combination with other features for the prognostication of cutaneous and subcutaneous canine MCTs (Romansik et al., 2007; Elston et al., 2009; Kiupel et al., 2011; Thompson et al., 2011).

The problem in case of this count is that there is no proper cut-off value for the exact prognostication of MCTs. Different researchers developed their different cut-off values. The other problem was that no study defined the total field area. It was proposed that high MC was related with decreased survival time in dogs. The MC for the cutaneous and subcutaneous MCTs is different. In a study it was reported that MC of more than 5 is related to decreased survival time in dogs with cutaneous MCTs. The MC of more than 4 is also related to decreased survival time in dogs with subcutaneous MCTs (Romansik et al., 2007; Kiupel et al., 2011; Thompson et al., 2011). Three tier system was used in a study and the dogs had cutaneous MCTs. The tumors revealed MC of more than 5 and the survival time was recorded only 2 months. In contrast, the survival time was recorded 70 months in the dogs having MC of less than 5.99 dogs revealed MC more than 5 and 80 dogs revealed MC less than 5 in that study.

The correlation of MC with the metastasis, prognosis and the survival time has been reported in that study. MC is also related to the grades of MCTs because 7 dogs having grade 2 MCTs and MC of more than 5 revealed survival time of 2 months. On the other hand, 72 dogs with grade 2 MCTs and having MC less than 5 revealed survival time of 80 months. 3 dogs of grade 3 MCTs having MC of less than 5 revealed survival time of less than 18 months (Romansik et al., 2007). Only 3 dogs revealed this different thing. It was thought that MC could be used to identify less aggressive high grade MCTs. The similar results were recorded in another study in which 3 out of 10 dogs with high grade MCTs and low MC were died due to MCT associated diseases. In that same study the dogs having low

grade MCTs and MC of less than 2 also died due to MCT associated diseases (Kiupel et al., 2011). The study of subcutaneous MCTs also revealed similar results. The 5 out of 12 dogs having MC of more than 4 revealed metastasis (Thompson et al., 2011). The lower number of MC is indicating that aggressive cutaneous and subcutaneous may also have low MC. The grades and the MC should be preferred for the investigation of MCTs. For the use of therapeutics these parameters should be measured correctly. Diagnostic sensitivity, specificity, and accuracy have been reported in a study of canine MCTs on the basis of Ki67 index and MC. 95 cutaneous MCTs were used in that study and no grades were identified only grade 2 was examined (Berlato et al., 2015). The specificity for the identification of high grade MCTs was recorded at 95% when grade 2 MCTs were determined.

The percentage of specificity with Ki67 index determination was 91% when it was recorded without determination of grades of tumors. The accuracy rates for the diagnosis were also calculated and these were 88% and 79%, respectively. According to that study these proliferation markers are important for the accurate prognostication of both cutaneous and subcutaneous MCTs (Berlato et al., 2015).

1.12.2. KIT Expression and C-kit Mutation

Prognostication of MCTs is very important for determining the accurate survival time of dogs. KIT plays an important role in the diagnosis, prognosis and determination of survival time of dogs. C-kit mutations are involved in the development of MCTs. The ITD mutation is also due to the involvement of c-kit mutation. This mutation takes place on exon 11. In a study it was stated that KIT expression in neoplastic mast cells may be a negative indicator for prognostication of MCTs (Zemke et al., 2002; Webster et al., 2006; Takeuchi et al., 2013). While different kinds of mutation have been identified like deletion and duplication. The mutation on exon 11 was considered the most important mutation and 20-30% of dogs revealed this mutation in the cutaneous MCTs (Zemke et al., 2002; Downing et al., 2002; Webster et al., 2008). The other mutations were also identified in exons 8 and 9. These

mutations are not common in canine MCTs. it has been reported that only 5% of MCTs revealed these kinds of mutations. These mutations are also not associated with the prognostication of MCTs (Letard et al., 2008). Eighty-six dogs were used in a study and 30 dogs revealed c-kit mutation in exon 11 and all of these tumors were high grade cutaneous MCTs. Seventeen MCTs out of remaining 56 were of low grade and had Ki67 index more than 23 and more than 56 combined score of AgNOR × Ki67. Twelve out of those thirty MCTs also revealed Ki67 index more than 23 and more than 56 combined score of AgNOR × Ki67. Only 5 out of 56 dogs revealed mutation in the exon 8 and were high grade MCTs. C-kit mutation is related to high grade MCTs and also indicates the decreased survival time of dogs. It also indicates the development of MCTs related mortality and increased chances of reoccurrence of MCTs in dogs.

The chances of recurrence of MCTs are twice as high in the dogs showing mutations in exon 11 as compared to other mutations (Zemke et al., 2002; Webster et al., 2006; Takeuchi et al., 2013). Nineteen out of forty-nine dogs revealing mutation in exon 11 died within 1 year. The cause of the death was the MCT associated diseases. On the other hand, only 5 out of 40 dogs died revealing mutation in exon 8 due to MCT related diseases. Therapeutic studies were also reported and it was stated that prednisolone and vinblastine had no beneficial effect in the dogs revealing mutations in exon 11 (Webster et al., 2008). It indicated that mutation in exon 11 is a good predictor for the accurate therapeutics against MCTs (Isotani et al., 2008; London et al., 2009; Nakano et al., 2014). Tyrosine kinase inhibitor therapy is considered effective against MCTs revealing mutation in exon 11.

Dogs with MCTs revealing mutation in exon 11 had response rates that were approximately twice as high compared to dogs having MCTs with wild-type c-kit after treating with toceranib (London et al., 2009). The process of gel-based PCR is the process for its identification. This process also helps to predict metastasis and reoccurrence of MCTs in dogs (Zemke et al., 2002; Webster et al., 2006). PCR is really important for the determination of accurate therapy especially for tyrosine kinase inhibitor therapy. Aberrant kit expression has also been associated with the negative prognosis of MCTs in dogs

especially cutaneous MCTs (Reguera et al., 2000; Kiupel et al., 2004; Preziosi et al., 2004; Da Costa et al., 2007).

There are two localized expression (membranous and cytoplasmic) of KIT that have been reported previously (Reguera et al., 2000; Kiupel et al., 2004) in high grade canine cutaneous MCTs. KIT expression was described in three specific patterns. The first staining pattern was peri-membranous (pattern I), second staining pattern was stippled cytoplasmic (pattern II) and the third staining pattern was diffused cytoplasmic (pattern III) (Kiupel et al., 2004). The last two patterns were associated with the less survival time and also high chances of recurrence. These findings were independent from the mutation of c-kit in exon 11. The chemotherapy (vinblastine and prednisolone) results showed that pattern III MCTs had less survival time as compare to pattern II MCTs. The DFIs were also less in the pattern III MCTs as compared pattern II MCTs (Kiupel et al., 2004). Increased cytoplasmic and membranous KIT expressions were found in the high grade MCTs but there was no difference between focal and diffuse cytoplasmic staining (Da Costa et al., 2007). The assessment of association between survival endpoints and KIT immunoreactivity was not done. Most subcutaneous MCTs were not able to show a c-kit mutation on exon 11 but the KIT localized patterns were associated with local recurrence and metastasis in canine subcutaneous MCTs (Thompson et al., 2011). Subcutaneous MCTs showing KIT pattern II and III were at high risk of local recurrence and metastasis. Chances for the recurrence were 88% and metastasis were 92%. The KIT method was a more sensitive method for the prediction of local recurrence and metastasis in MCTs as compared to the MC method (Thompson et al., 2011).

1.12.3. Some Other Molecular Markers in Canine MCTs

Many different molecular markers (p53 expression, MDM2 expression, serotonin and serotonin receptor expression, COX-2 expression, cyclin D1 expression, prostaglandin E2 expression, plasma histamine concentrations, and p21 expression) have been reported in canine MCTs (Ginn et al., 2000; Ishiguro et al., 2003; Wu et al., 2004 ; Wu et al., 2006;

Fröberg et al., 2009). The correlation between these markers, grades of tumors, staging and survival time has also been reported. These molecular markers also provide similar findings as previous markers. There was no new information added by these markers. The expression of minichromosome maintenance protein 7 (MCM7) has been reported in previous study (Berlato et al., 2012). A cut-off value of more than 0.18 was found significant for the confirmation of less survival time in MCTs. The survival time was 187 days regardless of grade of MCTs. The MCTs showing cut-off value of MCM7 less than 0.8 were able to show survival time of more than 600 days (Berlato et al., 2012). The cytological examination with fine needle aspiration method was thought to be a good method for the initial diagnosis of MCTs.

The cytological method was able to diagnose almost 80-90% cases. After that incisional biopsy was thought to be preferred method but due to increased costs and complication of wounds, it was not adopted regularly. Enlarged lymph nodes were found in some cases and it was suggested that cytological examination of these lymph nodes be done. Abdominal ultrasound was recommended in the dogs showing clinical signs of vomiting and melena. Cytological method can give initial diagnosis but grading is necessary for the confirmation of therapy. Excisional biopsy was preferred to remove the MCTs completely after the evaluation of MCTs with cytological methods in case of cutaneous MCTs. Surgical removal should be the first cure and should be done properly (Meuten, 2017). For the MCTs of 4 cm diameter, the lateral margin of 1cm and deep margin of 4mm were taken during surgical removal. If tumors were showing more aggressive behavior, then the lateral margin of 3cm was taken. Complete margin evaluation should be done for the proper prognostication and selection of therapy. No need of local additional therapy in the MCTs having clean margins. When tumor is low grade but margins are not clean then additional local therapy is not recommended after the evaluation of proliferation markers (KIT, Ki67, and Ki67xAgNOR). In the presence of less expression of proliferation markers in the tumor having dirty margins there was no need of additional local therapy. The low grade or some high grade MCTs had dirty margins and also showed higher expressions of proliferation markers then additional local therapy was not considered.

For the high grade MCTs showing incomplete or narrow margins, local therapy was suggested. For the selection of systemic therapy evaluation of histological grades, proliferation markers, PCR for the confirmation of c-kit mutation (exon 11 or others) and the KIT expressions with immunohistochemistry were done. For the high grade cutaneous MCTs showing metastasis and aggressive behavior, systemic therapy was considered. Systemic therapy was not considered in low grade MCTs showing no metastasis, less expression of proliferation markers (Ki67, Ki67xAgNOR), no c-kit mutation and also showing KIT pattern I. The systemic therapy was considered in the low-grade MCTs which had no metastasis but showed high expression of proliferation markers. The cutaneous MCTs showing metastasis was considered for systemic therapy. In the low grade MCTs which were not showing metastasis but whose c-kit mutation was positive on exon 11 and also showed KIT pattern of II or III, the systemic therapy was considered significant (Sledge et al., 2016; Meuten, 2017).

1.12.4. Osteopontin

Osteopontin (OPN) is an extracellular matrix protein. Bone sialoprotein 1 (BSP-1), secreted phosphoprotein 1 (SPP1), and early T lymphocyte activation 1 (ETA-1) are the different names of OPN. These different names indicate that OPN has multiple functions (Vaschetto et al., 2008; Clemente et al., 2016). The identification of OPN took place in the mineral ECM (extra cellular matrix) of bone, as a major sialoprotein (Franzen and Heinegard, 1985; Fisher et al., 1987; Prince et al., 1987; Zhang et al., 1990). Its name was introduced to show the potential role of the bone protein. It can act as a bridge between cells and hydroxyapatite (Oldberg et al., 1986). The name of “Eta-1” was evolved from the identification of lymphocytes and macrophages activation. SPP1 was also given to indicate the broader functional role of OPN (Fet et al., 1989; Craig and Denhardt, 1991).

In the experimental studies, downregulation of OPN expression was related to reduce growth in soft agar, growth of injected cells as primary tumors and experimental metastasis

(Denhardt et al., 1994; Denhardt et al., 2001). Reduction in the tumorigenecity of (Hepatocyte Growth Factor) HGF-transformed cells was indicated by the downregulation of OPN. It also implicated that OPN play a critical role in the transformation process of HGF (Ariztia et al., 2003). In experimental study, reduction in the growth of primary tumors and metastasis was reported due to the downregulation of OPN (Wu et al., 2000). In another experimental study, the role of OPN was explained in the experimental metastasis. It was stated that the OPN expression was able to convert the benign tumor cells to the complete metastatic form (Barraclough et al., 1998). It was interesting in that experiment that the DNA fragments that were responsible for all this process, were not able to code OPN directly. They were not even able to code for any other protein. These DNA fragments acted as the competitors and facilitated the binding of transcription factors TCF-4. These transcription factors were the main components of the downregulation of OPN. It was stated that increased expression of OPN is related to the malignancy of different tumors. The association of increased OPN expression with metastatic phenotypes has been reported, and these metastatic phenotypes were responsible for selection and expression of different kind of breast cancer cells in humans (Urquidi et al., 2002; Kang et al., 2003). OPN expression in normal cells and tumor cells revealed differential effects.

A model study of squamous cell carcinoma was conducted in mice and the tumors revealed more malignancy in the rats with deficient OPN. The metastasis of tumor in lungs were also numerous in the rats having deficient OPN (Crawford et al., 1998). In this study, the host cells expressed the really important role of OPN in tumorigenesis. The development of primary tumors was quite similar in all the rats with or without deficient OPN. These tumors did not actually express the proper effect of OPN on the metastasis (Feng and Rittling, 2000; Chen and Rittling, 2003). In other experimental study, melanoma cells were used and they revealed weak expression of OPN and a lower number of metastases in the mice with deficient OPN (Nemoto et al., 2001). According to the results of these experimental studies it is clear that OPN can play important role in the tumor development and that it can be affected by different parameters.

These parameters may include the type of tumor and the experimental system. It can also reflect the important activity of tumor microenvironment for the determination of OPN effect. It has been suggested that the different cells in microenvironment of tumors may produce OPN that has ability to play different functions. These cells of tumor microenvironment include immune cells, remodeling blood vessels, bone cells or the tumor cells themselves. It can be suggested that OPN from different sources has different functions. It was stated in the example that OPN originating from different cells may have ability to differentiate post-transitional changes or may be differential cleavage. It suggested that OPN has different functions depending on its source of origin. An experimental study was conducted for the expression of OPN and total of 116 cases of medullary thyroid carcinoma were used. The 91 out of 116 revealed positive OPN expression. C-cell hyperplasia and thyroid parenchyma of the tumor also revealed high expression of OPN. The different isoforms of OPN (OPNa, OPNb, OPNc) also revealed same expression in the tumor cells. They concluded in that study OPN expression is different in the medullary thyroid cancer as compared to other tumors. It was suggested that OPN expression is related to the good prognosis of medullary thyroid cancer (Ferreira et al., 2016).

1.12.5. VEGF

Vascular endothelial growth factor (VEGF) regulates the process of angiogenesis in normal organs and also in different diseases (Siemeister et al., 1998; Neufeld et al., 1999; Veikkola et al., 2000; Giles, 2001; Ferrara et al., 2003). Different roles of VEGF have been reported in different studies. One of its roles is mediator for the angiogenesis in different diseases. The other role of VEGF is autocrine growth regulation in different tumors (Siemeister et al., 1998; Veikkola et al., 2000; Giles, 2001; Gerber and Ferrara, 2003; Shinkaruk et al., 2003). The neoplastic cells have ability to express VEGF and its receptors (Giles, 2001; Gerber and Ferrara, 2003; (Shinkaruk et al., 2003). VEGF and its receptors expressions have also been reported in many canine tumors. It also has been discussed that VEGF and

its receptors were the target of drug therapy (Restucci et al., 2004; Wergin and Kazer-Hotz, 2004; Wergin et al., 2004).

Close association of mast cells and blood vessels has been found. Mast cells are also found at the sites of angiogenesis (Yano et al., 1999). These mast cell rich sites are the considered the periphery of solid tumors (Tth et al., 2000; Imada et al., 2000). Mast cells contribution to the angiogenesis process has been reported in many studies (Yano et al., 1999; Coussens et al., 1999; Ribatti et al., 2002; Walgenbach et al., 2002).

1.12.6. Oct-3/4

Oct-3/4, also known as POU5F1, is a transcription factor. It plays an important role in the regulation of pluripotency in the initial stages of mammalian development (Okamoto et al., 1990; Rosner et al., 1990). Embryonic stem cells require sufficient amount of Oct-3/4 for sustaining the self-renewal process. The changes in the critical amount of Oct-3/4 induce divergent cell fates. The expression of Oct-3/4 in murine and human embryonic stem cells has been reported. Different kinds of Oct-3/4 expressions have also been found in the different solid tumors of humans (Jin et al., 1999; Ezeh et al., 2005; Atlasi et al., 2007). It expression has also been reported in testicular germ cell tumors (Looijenga et al., 2003). It is great possibility that the expression of Oct-3/4 may play an important role in the malignancy process and cancer stem cell research. Oct-3/4 is considered the stem cell marker because positive expressions of Oct-3/4 have been reported in pluripotent cells (Liedtke et al., 2008). The Oct-3/4 expression has been used in many studies to evaluate the diagnosis, prognosis and for the selection of chemotherapeutics (Raman et al., 2006; Saigusa et al., 2009; Vargas et al., 2015; Meesuwan et al., 2021).

1.12.7. TNF-α

TNF-α is one of the important inflammatory cytokines. There are different sources of TNF-α including immune cells, macrophages and T-cells. Fibroblasts and tumor cells can also produce TNF-α (Wajant et al., 2003). The TNF-α also have an antitumoral effect. It can increase the permeability of tumor vessels. Due to increased permeability the tumor cells can enter in blood easily. It also increases the accumulation of melphalan and it play antitumoral role together with melphalan (van Horssen et al., 2006). The TNF-α also play important role in the hepatic carcinogenesis together with NFkB. The activation of both of these is an important factor for the acceleration of tumor progression (Pikarsky et al., 2004). The role of TNF-α has been found in the gastrointestinal carcinogenesis and it also has been expressed in the biopsies of colorectal cancer and ulcerative colitis (Popivanova et al., 2008). The important role of TNF-α in the skin, hepatic and gastrointestinal carcinogenesis has been reported in previous study (Kovacevic et al., 2008). Mast cells have ability to promote recruitment of leukocytes at different stages (Flier et al., 1993). The mast cells of mouse (both in vitro-derived and mucosal) produce TNF-α (Gordon and Galli, 1990; Gordon and Galli, 1991). TNF-α secreted from the mast cells and play important role in the fibroblast collagen expression.

2. MATERIALS AND METHODS

2.1. Chemicals, Kits and Solutions

Formaldehyde 37% w/v (CARLO ERBA) Batch number: V0F0072301
Alcohol absolute anhydrous (CARLO ERBA) Batch number: V0A067280A
Xylene (VWR chemicals) Lot number: 19L054008
Paraffin wax (Histomed) Lot number: 032022.1440506
Hematoxylin (C.1.75290) (MERCK®)
HCL (109060.1000) (MERCK®)
Acetic acid (glacial) (100063.2511) (MERCK®)
Eosin Y disodium salt (E4382-25G) SIGMA-ALDRICH®
Entellan™ (1.07960.0500) (MERCK®)
Hydrogen peroxide 30% (CARLO ERBA) Batch number: V0H0721701
Sodium citrate tribasic ehydrate (S4641) (SIGMA-ALDRICH®)
Trizma Base (T1503) (SIGMA®)
Citric acid monohydrate (C1909) (SIGMA-ALDRICH®)
Sodium chloride (27810.295) (VWR chemicals)
Sodium phosphate monobasic (71496) (Fluka Analytical)
Sodium phosphate dibasic (S5136) (SIGMA®)
Albumin Bovine serum (A2153) (SIGMA®)
AEC Substrate System Chromogen (red) (Thermo Scientific)
ABC HRP Kit. (Peroxidase) premium (VECTASTAIN® Elite®)
Magenta color chromogen (ImmPACT Vector Red (magenta) SK-5105)
APES (3-Aminopropyl triethoxysilane) (A3648) (Sigma-Aldrich)
Glycerol (W252506) (Sigma-Aldrich®)

2.2. Instruments and Apparatuses

Biopsy paraffin cassettes (074.04.003) (Isolab)

Microscope slides (Isolab)

Microscope cover glasses (0680) (Thermo Scientific)

Tissue processor (Leica TP1020)

Paraffin dispenser (Leica EG1120)

Refrigerator (BK8300T-BEKO)

Rotary microtome (Leica RM2155)

Tissue floating bath (Leica HI 1210)

Microtome blades (FEATHER®)

Incubator (JSGI-50T)

Microwave oven (MWZ5-BX-VESTEL)

Hydrophobic barrier pen (ImmEdge™ Vector Labs)

Humidity chamber (040204) (Biolab)

Micropipette (0.5-20 ul) (WITEG Germany)

Micropipette (5-50 ul) (Transferpette®S)

Micropipette (20-200ul) (Transferpette®S)

Micropipette (100-1000ul) (Transferpette®S)

Micropipette (1-10ml) (Transferpette®S)

Lab tips blue (100-1000ul) (Thermo Scientific)

Lab tips yellow (5-200ul) (Thermo Scientific)

Lab tips white (0-5ul) (Thermo Scientific)

Eppendorf tubes (Isolab Germany)

Surgical face masks (Kozmax)

Surgical gloves (Valeria)

Measuring cylinders (Isolab Germany)

Light Microscope (Zeiss Axio Lab.A1)

Camera (AxioCam ICc 5)

2.3. Materials

Material for the thesis study was taken from the archives of the Department of Pathology. The laboratory of the pathology department is working commercially to help out the

different hospitals and clinics regarding the diagnosis of different diseases. These hospitals and clinics are mostly sending tumor samples of different organs. Sixty samples of canine cutaneous MCT were used in this thesis study. The details of the cases are given in Table 2.1. The ethical approval for this study was also taken from the Afyon Kocatepe University Animal Experiments Local Ethics Committee, Afyonkarahisar, Türkiye. The number of the ethical report was AKUHADYEK-22-21. Tumor samples arrived in the 10% buffered formalin solution. After the fixation in formalin solution, cutting of tumor samples was done with disposable scalpel and biopsy cassettes were prepared. These biopsy cassettes were again put in the 10% buffered formalin solution for the fixation of inner part of the tumor. Sometimes tumors are bigger and formalin cannot enter inside the tumor tissues. These bigger tumors were cut into pieces and biopsy cassettes were made, which were again put in the formalin solution for proper fixation. Transvers and longitudinal sectioning methods were used for the cutting of tumors. Transversal and longitudinal sectioning help in the proper examination of tumor borders. After the fixation in formalin solution, processing of tissues was done in the tissue processor. Overnight processing method was used. Tissue processor contains 12 different compartments. First three compartments were used for distilled water, five compartments for alcohol, two compartments for xylene, and two compartments were for paraffin wax.

The biopsy cassettes were kept in the tap water compartments for 30 minutes. The five compartments after the tap water contain alcohol solutions of different percentages. First compartment of them contains 70% alcohol solution and tissues were waited for 1 hour in that compartment. The second compartment contains 80% alcohol solution and tissues were waited for one and half hour in that section. The third compartment contains 90% alcohol solution, and the tissues were waited for 1 hour in that compartment. The fourth and fifth compartments contain 100% alcohol solution, and the tissues were kept for an hour and half in each of those compartments. After the alcohol compartment, there were two compartments for xylene solutions. The tissues were waited for an hour and a half in each of those xylene compartments. Two last compartments were for paraffin wax solution. The temperature of these compartments was 58-62°C and the paraffin wax was in melted form

in that compartment. The tissues were waited for an hour and a half in each of those paraffin wax compartments. Tissue blocking was performed following the completion of tissue processing. A paraffin dispenser was used for the melting of paraffin wax. After the blocking of tissues, the blocks were put in the refrigerator because the cold blocks are easy to trim.

Table 2.1: The detail of cases.

Laboratory case number	**Thesis case number**	**Breed**	**Sex**	**Age (years)**
2982-19	1	French Bulldog	Female	11
2860-19	2	Labrador	Male	10
2817-19	3	Golden Retriever	Female	13
2810-19	4	Golden Retriever	Female	10
2804-19	5	Pug	Male	08
2798-19	6	Dogo Argentino	Male	10
2756-19	7	Golden Retriever	Female	08
2735-19	8	Labrador	Female	11
2662-19	9	Golden Retriever	Female	12
2657-19	10	English Pointer	Female	11
2569-19	11	Golden Retriever	Male	10
2567-19	12	Siberian Husky	Male	09
2490-19	13	Terrier	Female	12
2499-19	14	Mix	Female	05
2381-19	15	Cane Corso	Male	10
2254-19	16	Golden Retriever	Female	12
2239-19	17	Golden Retriever	Female	11
2228-19	18	Mix	Female	04
2208-19	19	Golden Retriever	Male	19
2194-19	20	Labrador	Female	08
2181-19	21	French Bulldog	Male	08
2098-19	22	Golden Retriever	Female	10
2103-19	23	American Bulldog	Male	10
2065-19	24	Mix	Female	09
1994-19	25	Golden Retriever	Female	09
1915-19	26	Pug	Female	03
1896-19	27	Golden Retriever	Female	10
1821-19	28	Dogo Argentino	Female	13
1795-19	29	Boxer	Female	09
1660-19	30	Pug	Male	04

1632-19	31	Samoyed	Female	11
1460-19	32	German shepherd	Male	06
1202-18	33	Siberian Husky	Male	11
1170-18	34	Golden Retriever	Female	10
1118-18	35	Mix	Male	06
850-18	36	American Pit Bull Terrier	Male	06
797-18	37	Dogo Argentino	Male	11
763-18	38	Cocker Spaniel	Female	08
755-18	39	Golden Retriever	Male	12
732-18	40	Jack Russel Terrier	Male	08
721-18	41	Golden Retriever	Male	13
707-18	42	Golden Retriever	Male	14
626-18	43	Golden Retriever	Female	03
611-18	44	Mix	Female	10
MCT-20	45	Dogo Argentino	Female	10
MCT-24	46	Golden Retriever	Female	08
MCT-25	47	Mix	Female	08
MCT-89	48	Mix	Male	07
MCT-91	49	Golden Retriever	Male	11
MCT-95	50	Golden Retriever	Male	09
1921-19	51	Dachshund	Male	09
MCT-100	52	Dachshund	Male	11
MCT-108	53	Golden Retriever	Female	11
MCT-120	54	Golden Retriever	Male	08
MCT-128	55	Golden Retriever	Female	10
MCT-117	56	Mix	Female	09
1189-18	57	Cane Corso	Male	10
322-18	58	Jack Russel Terrier	Female	08
284-18	59	Golden Retriever	Male	10
176-18	60	Dogo Argentino	Female	09

2.4. Methods

2.4.1. Histopathological Methods

Sections of 4 micron (μ) were taken from paraffin blocks using a microtome for histopathological and immunohistochemical examination. After getting the sections, the slides were incubated in the incubator for 2 hours at a temperature of 60°C. After the melting of the extra wax, the slides were taken out of the incubator for the HE staining. Slides were deparaffinized in the xylene solution for 20-30 minutes. After deparaffinization, slides were passed through graded alcohols. The alcohol solutions used had percentages of 100%, 90%, 80%, and 70%. After the washing with distilled water, slides were put in the Harris hematoxylin solution for 30 minutes. After the completion of staining process of slides with hematoxylin, the slides were put in an acid-alcohol solution for only 20-30 seconds to remove an extra hematoxylin. The removal of extra stain and the blue color of tissues were observed with the eyes, and then slides were put under the tap water for 20 minutes. After the tap water, the slides were washed with distilled water. After washing with distilled water, the slides were put in the eosin solution for 15 minutes. After the completion of eosin staining, the slides were washed with graded alcohol solutions. After the washing of slides with alcohols, they were cleared in xylene solutions for 15 minutes. After the completion of all these processes, the slides were cover slipped with the mounting medium. It is a ready-to-use, water free mounting medium. The tissues were examined under a light microscope. The diagnosis and the grading of MCTs was done using light microscope. The photos were taken by the camera fitted to the light microscope.

The diagnosis and grading of tumors were done according to the Patnaik (Patnaik et al., 1984) and Kiupel (Kiupel et al., 2011) grading systems with the help of HE stain. A MC was done in all the tumors according to different grades of canine cutaneous MCTs. The presence of necrosis was also considered significant in all the canine cutaneous MCTs. The process of margin evaluation was also done according to the method described in the previous study (Melo et al., 2021). The criteria for the clean margins were as follows: no tumor, no cluster or satellite cells near the margins, 4mm distance between tumor and normal skin and also measurement of radial and tangential tissues described in a previous study (Sledge et al., 2016). Other tumors that did meet these criteria were considered to

have dirty margins. The tumors for which the clinicians did not have complete excision information were not included in the process of margin evaluation.

2.4.2. Immunohistochemical Methods

Tissue sections of 4 microns were taken on special adhesive slides for the immunohistochemical staining. Tissues were deparaffinized with xylene and cleared with graded alcohol. Endogenous enzyme activity was quenched by treating the tissues with a 10% hydrogen peroxide solution for 10 minutes. A specified antigen retrieval with citrate buffer was done in steamer at 90°C for 15 minutes. Overnight incubation with primary antibodies for C-kit, Ki67, VEGF, OPN, Oct-3/4, and TNF-α, were done. The details of the primary antibodies are given in Table 2.2. After application of secondary antibodies, slides were incubated in a humidity chamber for 2 hours at room temperature (37°C). After washing the slides with buffer solution, streptavidin biotin peroxidase complex method (ABC-P) application was started. Biotinylated IgG was used and was incubated at room temperature for 1 hour. Finally, peroxidase conjugated avidin was dropped and allowed to react for 30 minutes at 37°C. Slides were washed with buffer solution, and tissues were treated with a red colored AEC peroxidase substrate. After completion of reaction, the slides were taken into distilled water and counter stained with Mayer's hematoxylin. Slides were covered with coverslips using an aqueous adhesive medium and examined under a light microscope.

Table 2.2: The detail of antibodies used in immunohistochemical analysis.

Primary	**Source**	**Species**	**Dilution**	**Monoclonal**

antibody				**/Polyclonal**
C-kit	A4502, DAKO	Rabbit	1/20	Polyclonal
OPN	Sc-21742, SANTA CRUZ BIOTECH.	Mouse	1/50	Monoclonal
Oct-3/4	Sc-5278, SANTA CRUZ BIOTECH.	Mouse	1/50	Monoclonal
VEGF	Sc-152, SANTA CRUZ BIOTECH.	Mouse	1/50	Monoclonal
TNF-α	Sc-52746, SANTA CRUZ BIOTECH.	Mouse	1/40	Monoclonal
Ki67	Sc-23900, SANTA CRUZ BIOTECH.	Mouse	1/25	Monoclonal

2.4.3. Statistical Methods

The descriptive analysis method was used for the statistical analysis. The correlation between categorical variables was investigated with the Chi-square test. The correlation between markers and other histopathological variables (grades of MCTs, presence of necrosis and MC) was also investigated with the use of the Chi-square test. SPSS software (18.00) was used for this statistical analysis. The significance level was 5% ($P<0.05$).

3. RESULTS

3.1. Macroscopic Findings

Different sizes of cutaneous MCTs were diagnosed ranging from few mm to 20 cm in this study. Nodular rashes to diffuse swellings or hairless, raised, erythematous to highly variable tumors were identified. Poorly circumscribed having ulcerated and pruritic surfaces, and show rapidly growing behavior were observed in most high-grade canine cutaneous MCTs.

3.2. Histopathological Findings, Diagnosis and Grading

Tumor samples were taken from thirty-three female and twenty-seven male dogs. Eighteen different dog breeds were presented. The detail of the number of dogs is as follows, twenty-three Golden Retrievers, 5 Dogo Argentinos, 3 Labradors, 3 Pugs, 2 French Bulldogs, 2 Siberian Huskies, 2 Cane Corso, 2 Jack Russel Terrier, 2 Dachshund, 1 Samoyed, 1 German Shepherd, 1 American Pitbull Terrier, 1 Cocker Spaniel, 1 English Pointer, 1 Terrier, 1 American Bulldog, and 1 Boxer . Eight dogs of mix breeds were also included in this study. The mean age of dogs was 9.25 years for the development of canine cutaneous MCTs. All the tumors were diagnosed and graded according to the Patnaik and Kiupel grading system for the cutaneous MCTs. The histopathological findings are given in Table 3.1. According to the Patnaik system, 17 dogs were diagnosed as grade 1, 33 dogs as grade 2 and 10 dogs as grade 3 MCTs. The percentages of grades were as follows, grade 1 (28.33%), grade 2 (55%) and grade 3 (16.67%). After that, the classification of tumors was done according to the Kiupel grading system. All the grade 3 tumors were high-grade tumors and all the grade 1 tumors were low-grade tumors. Twenty-five tumors from the grade 2 MCTs were graded as low-grade, and 8 tumors were graded as high grade MCTs. According to the Kiupel grading system, 70% tumors were graded as low grade and 30% were graded as high-grade canine cutaneous MCTs. The percentage of clean margins in canine cutaneous MCTs was 70% and 20% MCTs showed dirty margins. Other 10% MCTs were not clear because of the incomplete information provided by the clinicians.

Table 3.1: Histopathological findings in the canine cutaneous MCTs.

Grades of MCTs	**Location of MCTs**	**Size of cells**	**Shape of cells**	**Cytoplasm shape**	**Shape of nucleus**	**Number of nucleolus/ cells**	**Metachromasia**
1 (well differentiated) n=14	Superficial area of dermis	Uniform	Round to oval	Abundant	Round to polygonal/ oval	One or more than one	Marked
2 (intermediately differentiated) n=30	Superficial and deep dermis	Uniform/ anisocytosis/ multinucleation	Pleomorphic/ round to oval	Moderate/ abundant	Anisokaryosis/ round to oval	One or more than one	Moderate/ marked
3 (poorly differentiated) n=16	Superficial and deep dermis	Anisocytosis/ uniform/ multinucleation	Pleomorphic/ round to oval	Moderate/ scarce	Anisokaryosis /round to oval	One or more than one	Moderate/ marked

The depth of all the tumors was checked to confirm the cutaneous MCTs. Tumors were present in three areas, including the epidermis, superficial dermis and deep dermis. Rows of ribbons formed by the neoplastic cells were observed in different cases. Numerous eosinophils were found in many tumors, and some were like prominent neoplastic cells. Round to polygonal cells with round central to slightly eccentric nuclei were found on higher magnification. The cytoplasm of tumor cells showed a moderate to abundant amount, pale pink cytoplasm and contained granules that stained gray or blue with HE stain. Collagenolysis, sclerosis, edema, necrosis and lymphocytic infiltration were also found, and these findings may be due to secondary inflammation in the canine cutaneous MCTs. According to Patnaik and Bostock, well-differentiated tumors were graded as grade 1 (Fig 3.1A), intermediately differentiated tumors were graded as grade 2 (Fig 3.1B), and poorly differentiated tumors were graded as grade 3 (Fig 3.1C) canine cutaneous MCTs.

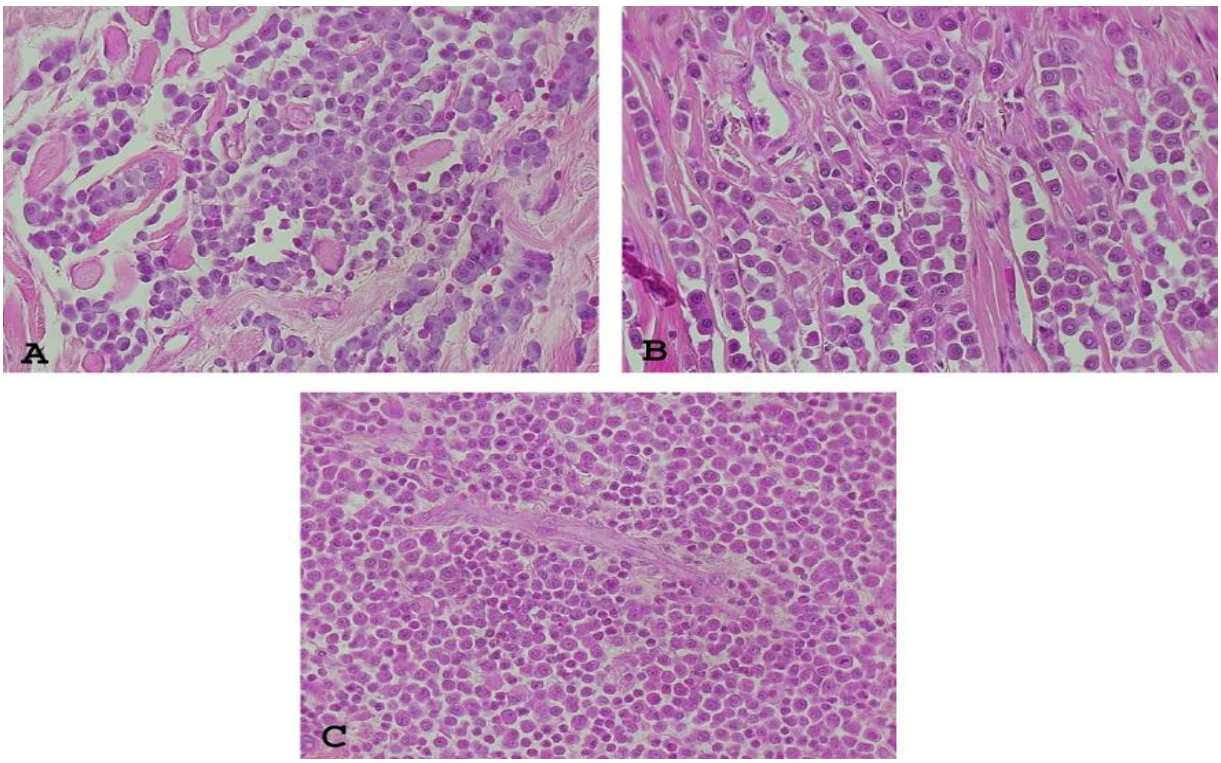

Fig 3.1: Patnaik grading system for canine cutaneous MCTs. (HE stain, 40x). A: Grade 1 (well differentiated) MCT. B: Grade 2 (intermediately differentiated) MCT. C: Grade 3 (poorly differentiated) MCT.

The grading system of Kiupel was also followed and the high-grade tumors showed 5-7 mitotic figures, 2-3 multinucleated cells, 2-3 bizarre nuclei, and karyomegaly in 10 HPF. The low grade (Fig 3.2A-B) canine cutaneous MCTs did not show these findings, and they were well circumscribed. Highest degree of anisokaryosis and mitotic activity was also found in high grade (Fig 3.2C-D) canine cutaneous MCTs.

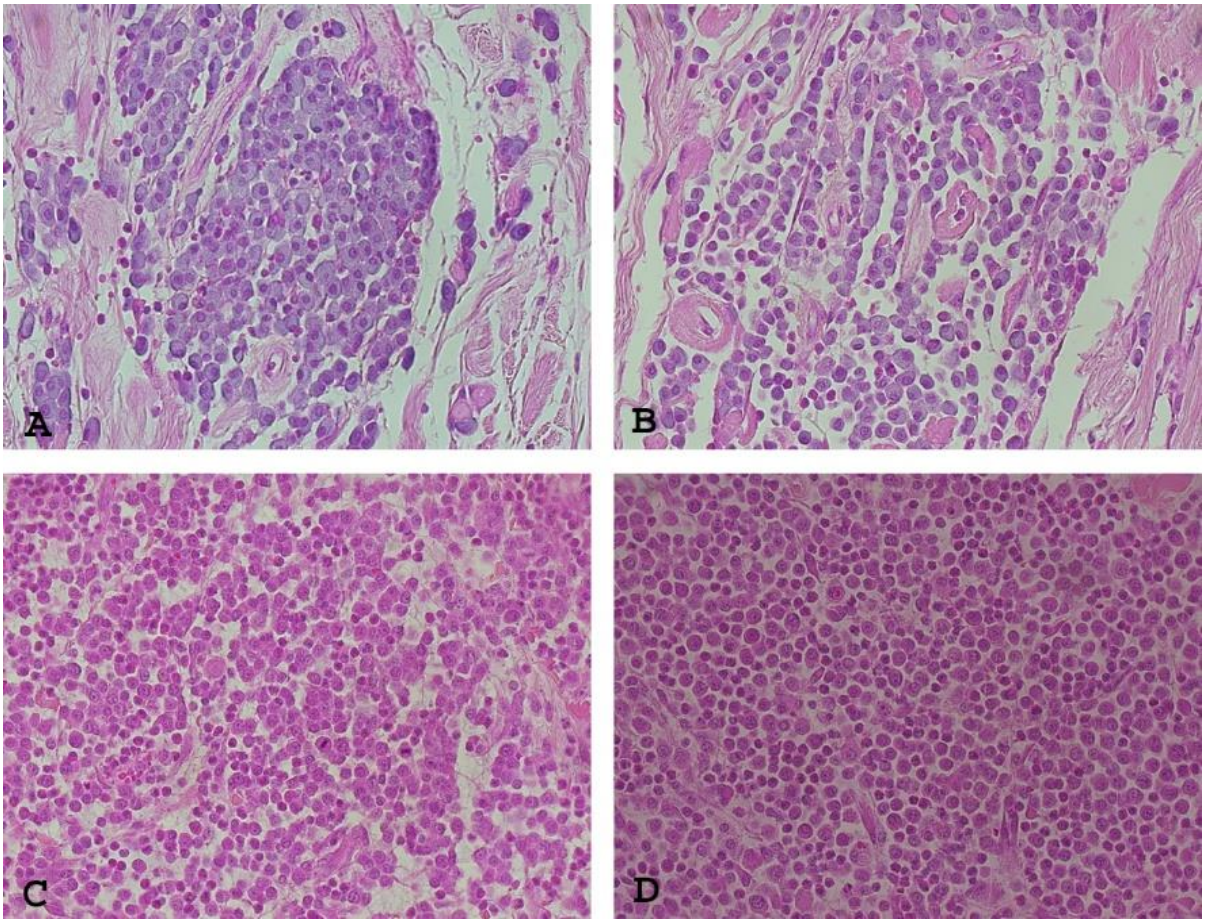

Fig 3.2: Kiupel grading system for canine cutaneous MCTs. (HE, 40x). A-B: Low grade MCTs. C-D: High grade MCTs.

3.3. Immunohistochemical Evaluation

3.3.1. Evaluation of KIT Immunopositivity

The evaluation of KIT immunopositivity was done according to the previously described method for MCTs. Three different patterns of KIT immunopositivity were evaluated in this study. The first pattern (I) showed perimembranous immunopositivity of KIT with minimal cytoplasmic immunopositivity. The second pattern (II) showed stippled cytoplasmic immunopositivity of KIT. The third pattern (III) showed diffused cytoplasmic immunopositivity of KIT. Thirty-two (53.33%) MCTs revealed membranous immunopositivity, 22 (36.67%) MCTs revealed stippled cytoplasmic immunopositivity and 6 (10%) MCTs revealed diffused cytoplasmic immunopositivity of KIT. The relation of KIT immunopositivity with the other histopathological variables was investigated with the Chi-square test, and significant ($P<0.05$) correlations were evaluated between KIT and grades of MCTs, MC and the presence of necrosis in this study (Table 3.2).

Table 3.2: KIT immunopositivity and other histopathological variables.

Grades of tumors	**Membranous KIT immunopositivity**	**Cytoplasmic KIT immunopositivity**	**Chi-square value**	**P-value**	**Total of rows**
Grade 1	12 (9.07) (37.50%)	5 (7.93) (17.85%)			17 (28.33%)
Grade 2	18 (17.60) (56.25%)	15 (15.40) (53.57%)			33 (55.00%)
Grade 3	2 (5.33) (6.25%)	8 (5.33) (7.14%)			10 (16.67%)
Total of columns	32 (53.33%)	28 (46.67%)	6.517	P<0.05	60
Necrosis					
No	29 (24.53) (90.62%)	17 (21.47) (60.71%)			46 (76.67%)
Yes	3 (7.47) (9.38%)	11 (6.53) (39.29%)	7.468	P<0.05	14 (23.33%)
Total of columns	32 (53.33%)	28 (46.67%)			60
Mitotic count per HPF					
0	23 (17.60) (71.87%)	10 (15.40) (35.71%)			33 (55.00%)
1	7 (6.40) (21.87%)	5 (5.60) (17.85%)			12 (20.00%)
2	1 (3.73) (3.12%)	6 (3.27) (21.42%)	13.318	P<0.05	7 (11.67%)
>4	1 (4.72) (3.12%)	7 (3.73) (25.00%)			8 (13.33%)
Total of columns	32 (53.33%)	28 (46.67%)			60

Grade 1 MCTs revealed mostly pattern I, grade 2 MCTs revealed all three patterns, and the grade 3 tumors revealed mostly pattern III, although some grade 3 tumors also revealed pattern II. Some grade 2 MCTs revealed both pattern II and III in the same tumor. Some grade 3 MCTs also revealed pattern II and III in one tumor. Low grade MCTs showed Pattern I and II but high grade MCTs showed pattern II and III. Some high grade MCTs revealed pattern II and III in the same tumor and the same thing was found in the low grade MCTs in which pattern I and II were seen in the same tumor. The presence of two patterns in the same tumor was quite interesting. The MCTs were classified on the basis of staining patterns. The cells in the form of clusters or groups were considered positive. The cells on the margins of tissues were not considered significant because of the possible artifacts. All the patterns are shown in Fig 3.3.

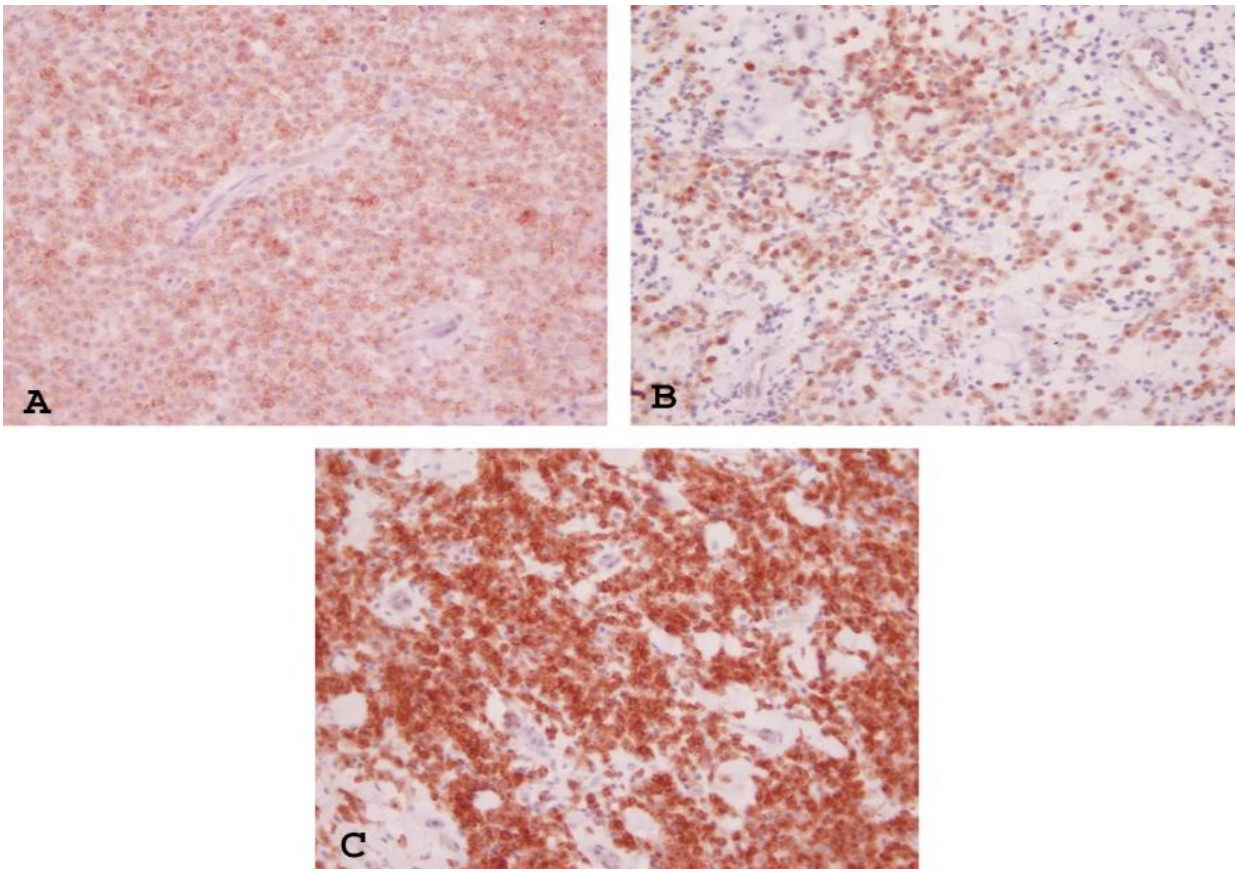

Fig 3.3: C-kit patterns evaluation. (ABC-P, AEC chromogen, Mayer's Hematoxylin counterstain, 40x). A: Peri-membranous staining (pattern I). B: Focal or stippled cytoplasmic with decreased membranous staining (pattern II). C: Diffuse cytoplasmic staining (pattern III).

3.3.2. Evaluation of OPN Immunopositivity

OPN was first time used in the history in case of canine cutaneous MCTs. Intensity of OPN expression was graded as nonspecific positivity (-), mild positivity (+), moderate positivity (++), diffuse positivity (+++), according to the method described in previous study. Eighteen MCTs showed (+), 17 MCTs showed (++) and 25 MCTs showed (+++) immunopositivity with OPN. The relation between OPN immunopositivity and grades of MCTs, presence of necrosis and MC was evaluated in this study. A significant relationship was found between OPN immunopositivity and grades of MCTs, presence of necrosis and MC (Table 3.3).

Table 3.3: OPN immunopositivity and other histopathological variables.

Grades of tumors	**OPN+**	**OPN++**	**OPN+++**	**Chi-square value**	**P-value**	**Total of rows**
Grade 1	2 (5.10) (11.11%)	4 (4.82) (23.52%)	11 (7.08) (44.00%)			17 (28.33%)
Grade 2	10 (9.90) (55.56%)	10 (9.35) (58.82%)	13 (13.75) (52.00%)			33 (55.00%)
Grade 3	6 (3.00) (33.33%)	3 (2.83) (17.64%)	1 (4.17) (4.00%)	9.692	P<0.05	10 (16.67%)
Total of columns	18 (30.00%)	17 (28.33%)	25 (41.67%)			60
Necrosis						
No	17 (13.80) (94.44%)	15 (13.03) (82.24%)	14 (19.17) (56.00%)			46 (76.67%)
Yes	1 (4.20) (5.56%)	2 (3.97) (11.76%)	11 (5.83) (44.00%)	10.420	P<0.05	14 (23.33%)
Total of columns	18 (30.00%)	17 (28.33%)	25 (41.67%)			60
Mitotic count/HPF						
0	14 (9.90) (77.78%)	12 (9.35) (70.59%)	7 (13.75) (28.00%)			33 (55.00%)
1	2 (3.60) (11.11%)	3 (3.40) (17.65%)	7 (5.00) (28.00%)			12 (20.00%)
2	1 (2.10) (5.55%)	1 (1.98) (5.88%)	5 (2.92) (20.00%)			7 (11.67%)
>4	1 (2.40) (5.56%)	1 (2.27) (5.88%)	6 (3.33) (24.00%)	13.530	P<0.05	8 (13.33%)
Total of columns	18 (30.00%)	17 (28.33%)	25 (41.67%)			60

Well differentiated MCTs showed diffuse cytoplasmic positivity with OPN, intermediately differentiated MCTs showed intermediate positivity with OPN but mostly poorly differentiated MCTs were not able to show proper positivity with OPN. OPN revealed a special pattern of diffuse cytoplasmic positivity in the low-grade and differentiated MCTs but the high-grade MCTs were not able to show such a kind of positivity with OPN. It means OPN expression can be very important for the three-tier grading system because the grade 1 (well differentiated) MCTs showed special diffused positivity with OPN (Fig 3.4A) and grade 2 (intermediately differentiated) MCTs also revealed intermediate positivity (Fig 3.4B). Non neoplastic mast cells also revealed positivity with the OPN in the high-grade MCTs but this positivity was less prominent in the malignant cells of grade 3 MCTs (Fig 3.4C).

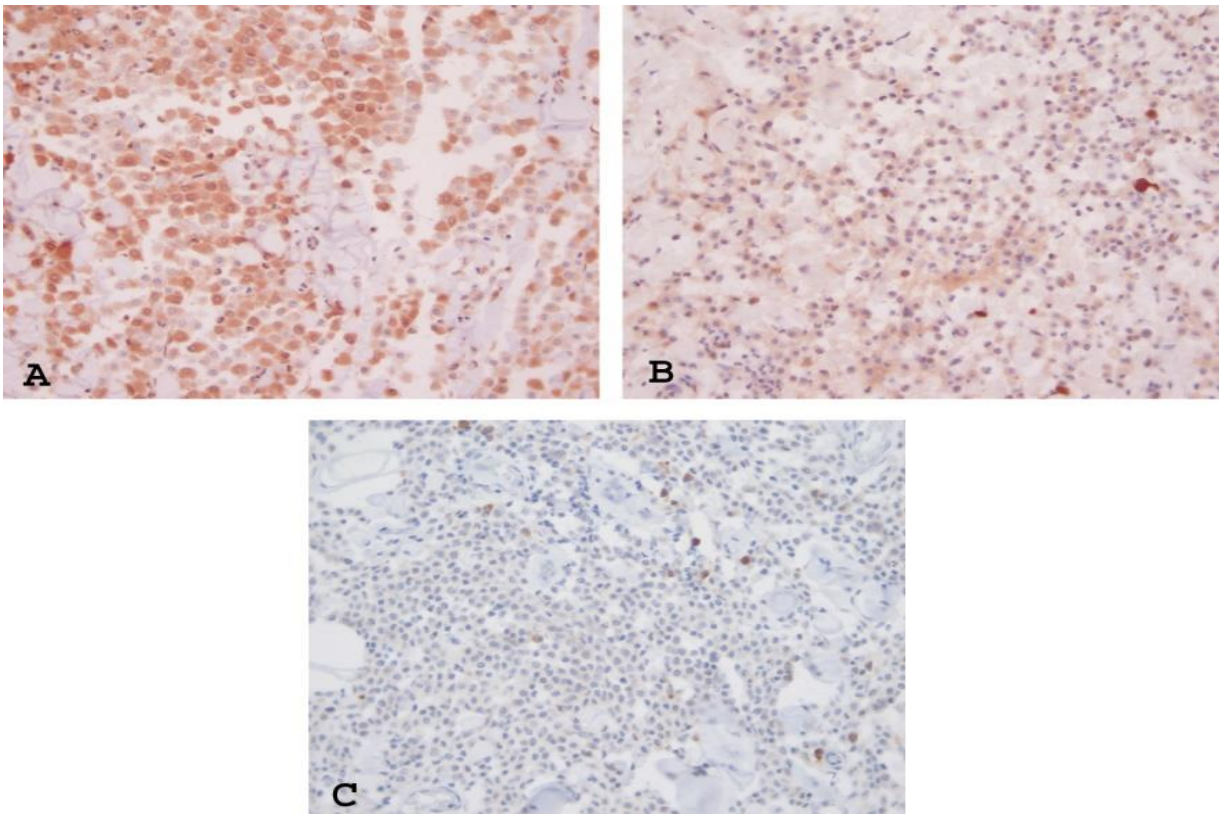

Fig 3.4: Evaluation of OPN immunopositivity. (ABC-P, AEC chromogen, Mayer's Hematoxylin counterstain, 40x). A: Well differentiated MCTs showed special diffused positivity. B: Intermediately differentiated MCTs showed intermediate positivity. C: Poorly differentiated MCTs were not able to show any positivity.

3.3.3. Evaluation of Oct-3/4 Immunopositivity

The intensity of Oct-3/4 expression was graded as nonspecific positivity (-), mild positivity (+), moderate positivity (++), and diffuse positivity (+++), according to the method described in a previous study. Positive cells in different areas of tumors were expressed as 0-25%, 25-50%, 50-75% and 75-100%. Eighteen MCTs showed (+), 16 MCTs showed (++) and 26 MCTs showed (+++) immunopositivity with Oct-3/4. The relation between OCT-3/4 immunopositivity and grades of MCTs, presence of necrosis and MC was investigated in this study. A significant correlation was found between OCT-3/4 and grades of MCTs, presence of necrosis, and MC (Table 3.4).

Table 3.4: Oct-3/4 immunopositivity and other histopathological variables.

Grades of tumors	**Oct-3/4+**	**Oct-3/4**++	**Oct-3/4**+++	Chi-square value	P-value	Total of rows
Grade 1	1 (5.10) (5.56%)	4 (4.53) (25.00%)	12 (7.37) (46.15%)			17 (28.33%)
Grade 2	10 (9.90) (55.56%)	10 (8.80) (62.50%)	13 (14.30) (50.00%)			33 (55.00%)
Grade 3	7 (3.00) (38.88%)	2 (2.67) (12.50%)	1 (4.33) (3.85%)	14.619	P<0.05	10 (16.67%)
Total of columns	18 (30.00%)	16 (26.67%)	26 (43.33%)			60
Necrosis						
No	17 (13.80) (94.44%)	14 (12.27) (87.50%)	15 (19.93) (57.70%)			46 (76.67%)
Yes	1 (4.20) (5.56%)	2 (3.73) (12.50%)	11 (6.07) (42.30%)	9.462	P<0.05	14 (23.33%)
Total of columns	18 (30.00%)	16 (26.67%)	26 (43.33%)			60
Mitotic count/HPF						
0	14 (9.90) (77.77%)	12 (8.80) (75.00%)	7 (14.30) (26.92%)			33 (55.00%)
1	2 (3.60) (11.11%)	2 (3.20) (12.50%)	8 (5.20) (30.78%)			12 (20.00%)
2	1 (2.10) (5.56%)	1 (1.87) (6.25%)	5 (3.03) (19.23%)			7 (11.67%)
>4	1 (2.40) (5.56%)	1 (2.13) (6.25%)	6 (3.47) (23.07%)	14.780	P<0.05	8 (13.33%)
Total of columns	18 (30.00%)	16 (26.67%)	26 (43.33%)			60

The immunohistochemical evaluation of Oct-3/4 was done according to the method described in a previous study. According to this method, the Oct-3/4 showed two different kinds of expressions in the canine MCTs of this study. The first expression was diffuse, and both the nucleus and cytoplasm showed positivity. Mostly well differentiated and intermediately differentiated MCTs (Fig 3.5A-B) showed this kind of expression. In second expression, only the cytoplasm showed positivity, but there were also some MCTs that did not show any positivity with Oct-3/4. Mostly undifferentiated or poorly differentiated MCTs showed second expression, in which some cells showed only cytoplasmic positivity and some cells were not able to show expression of Oct-3/4 (Fig 3.5C). The same two expressions were identified in low-grade and high grade MCTs. Most of the low-grade MCTs revealed first expression of diffused positivity. Most of the high grade MCTs revealed second expression involving cytoplasm only and some cells were also not able to show any expression. This finding of two expressions can be important for the grading of canine cutaneous MCTs.

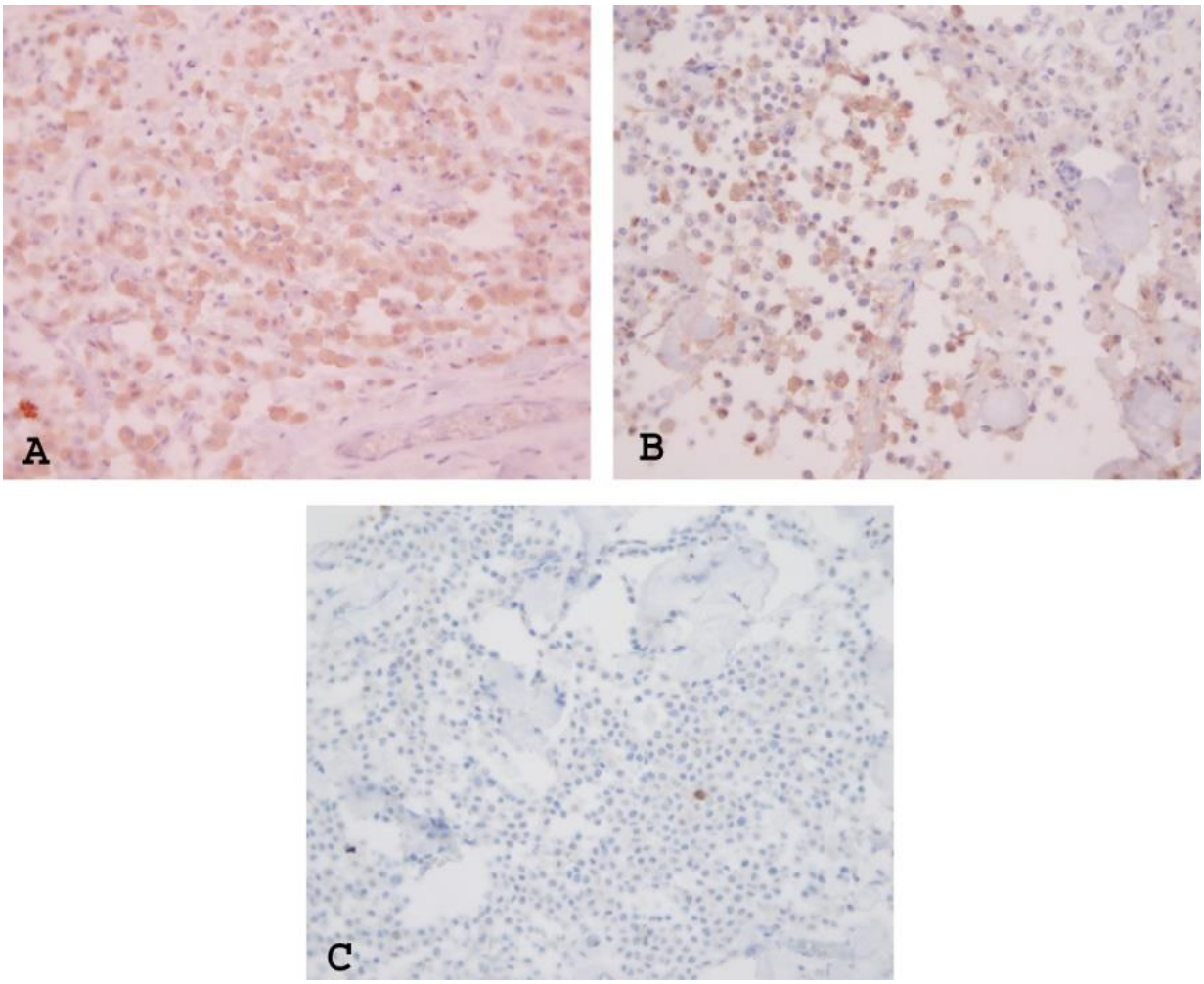

Fig 3.5: Evaluation of Oct-3/4 immunopositivity. (ABC-P, AEC chromogen, Mayer's Hematoxylin counterstain, 40x). A: Well differentiated MCTs showed special diffused (nuclear and cytoplasmic) positivity. B: Intermediately differentiated MCTs showed intermediate nuclear and cytoplasmic positivity. C: Poorly differentiated MCTs were not able to show any positivity.

3.3.4. Evaluation of Ki-67 Immunopositivity

The evaluation of Ki-67 immunopositivity was done according to the method described in previous studies. Ki-67 positive cells were counted manually. The largest portion of areas showing Ki-67 immunopositivity was selected using a 40x magnification of light microscope. The number of Ki-67 positive cells was counted in a 10 x 10 mm grid area. Five different field areas were selected, the number of Ki-67 positive cells were counted, and the average results of

Ki-67 positive cells were considered significant. Different percentages were calculated and 25% (+) Ki67 positivity showed cut-off value of 3.8, 50% (++) positivity revealed cut-off value of 7.6 and 75% and more positivity (+++) revealed cut-off values of 21.8 and more. Twenty-eight MCTs showed (+), 16 MCTs showed (++) and 16 MCTs showed (+++) immunopositivity with Ki67. The relation between Ki67 immunopositivity and grades of MCTs, presence of necrosis, and MC was investigated in this study. A significant correlation was found between Ki67 and grades of MCTs, presence of necrosis and MC (Table 3.5).

Table 3.5: Ki67 immunopositivity and other histopathological variables.

Grades of tumors	**Ki67**+	**Ki67**++	**Ki67**+++	Chi-square value	P-value	Total of rows
Grade 1	12 (7.93) (42.86%)	4 (4.53) (25.00%)	1 (4.53) (6.25%)			17 (28.33%)
Grade 2	15 (15.40) (53.57%)	8 (8.80) (50.00%)	10 (8.80) (62.50%)			33 (55.00%)
Grade 3	1 (4.67) (3.57%)	4 (2.67) (25.00%)	5 (2.67) (31.25%)	14.619	P<0.05	10 (16.67%)
Total of columns	28 (46.67%)	16 (26.66%)	16 (26.67%)			60
Necrosis						
No	25 (21.47) (89.29%)	14 (12.27) (87.50%)	7 (12.27) (43.75%)			46 (78.33%)
Yes	3 (6.53) (10.71%)	2 (3.73) (12.50%)	9 (3.73) (56.25%)	13.233	P<0.05	14 (21.67%)
Total of columns	28 (46.67%)	16 (26.66%)	16 (26.67%)			60
Mitotic count/HPF						
0	20 (15.40) (71.43%)	10 (8.80) (62.50%)	3 (8.80) (18.75%)			33 (55.00%)
1	6 (5.60) (21.43%)	3 (3.20) (18.75%)	3 (3.20) (18.75%)			12 (20.00%)
2	1 (3.57) (4.17%)	2 (1.87) (12.50%)	4 (1.87) (25.00%)			7 (11.67%)
>4	1 (3.57) (4.17%)	1 (2.13) (6.25%)	6 (2.13) (37.50%)	19.046	P<0.05	8 (13.33%)
Total of columns	28 (46.67%)	16 (26.66%)	16 (26.67%)			60

Two cut-off values for the identification of grading of canine cutaneous MCTs were identified. The cut-off value of Ki67<23 was considered significant for the low-grade and Ki67>23 was considered significant for high-grade canine cutaneous MCTs. Well differentiated MCTs revealed a very low number of Ki-67 positive cells (Fig 3.6A). Moderately differentiated MCTs showed moderate positivity (Fig 3.6B). Poorly differentiated MCTs showed a higher number of Ki-67 positive cells (Fig 3.6C).

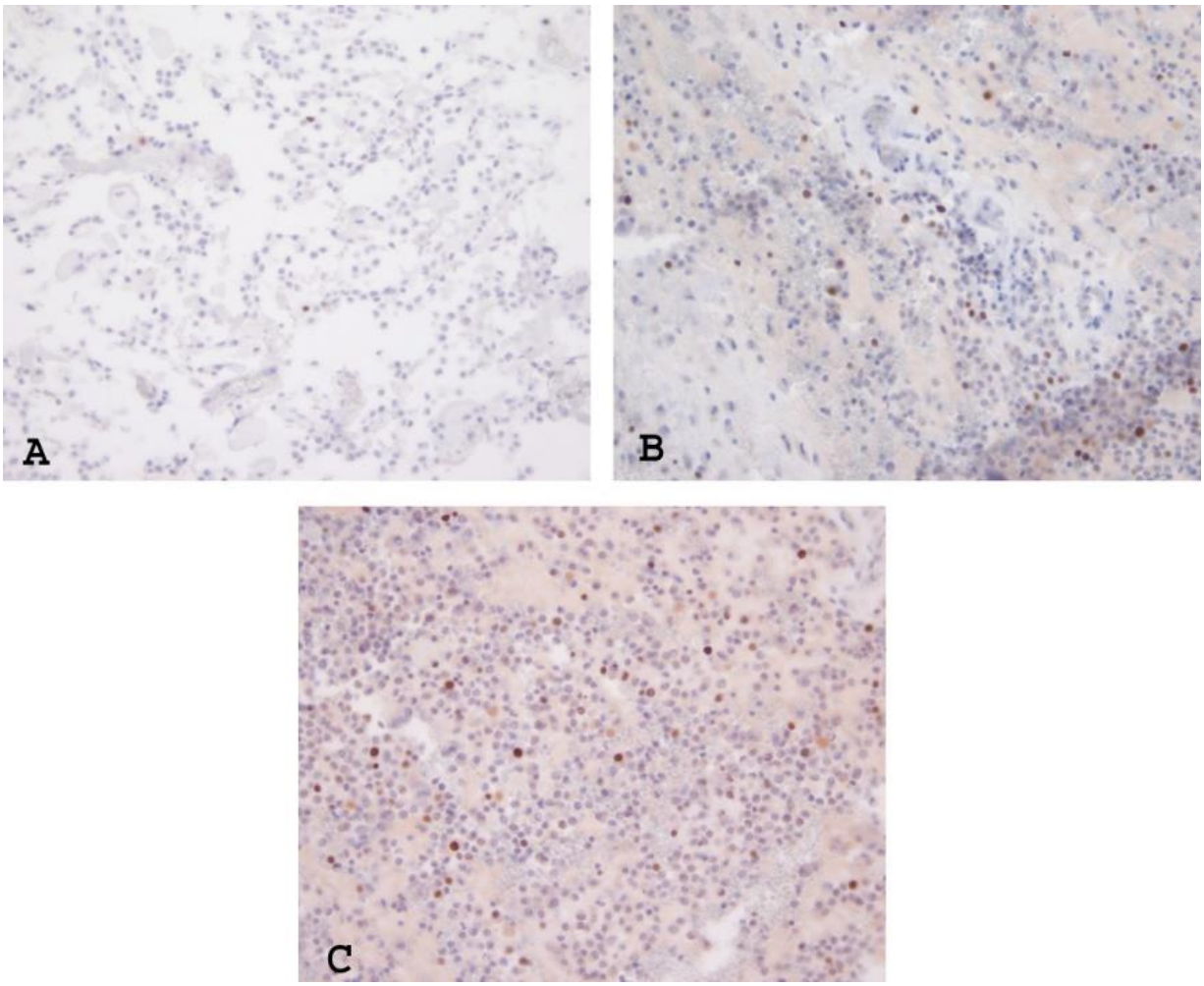

Fig 3.6: Evaluation of Ki-67 immunopositivity. (ABC-P, AEC chromogen, Mayer's Hematoxylin counterstain, 40x). A: Well differentiated MCTs revealed very low number of Ki-67 positive cells. B: Moderately differentiated MCTs showed moderate positivity. C: Poorly differentiated MCTs showed higher number of Ki-67 positive cells.

3.3.5. Evaluation of VEGF Immunopositivity

Two different methods were used to evaluate the expression of VEGF in canine cutaneous MCTs. In first method, intensity of VEGF expression was graded as nonspecific positivity (-), mild positivity (+), moderate positivity (++), diffuse positivity (+++), according to the method described in previous study (Melo et al., 2021). Positive cells in different areas of tumors were expressed as 0-25%, 25-50%, 50-75% and 75-100%. Twenty-six MCTs showed (+), 22 MCTs showed (++) and 12 MCTs showed (+++) immunopositivity with VEGF. The relation between VEGF immunopositivity and grades of MCTs, presence of necrosis and MC was investigated in this study. A significant correlation was found between VEGF and grades of MCTs, presence of necrosis and MC (Table 3.6).

Table 3.6: VEGF immunopositivity and other histopathological variables.

Grades of tumors	**VEGF**+	**VEGF** ++	**VEGF** +++	Chi-square value	P-value	Total of rows
Grade 1	9 (7.37) (36.61%)	2 (6.23) (9.09%)	6 (3.40) (50.00%)			17 (28.33%)
Grade 2	10 (14.30) (38.46%)	18 (12.10) (81.81%)	5 (6.60) (41.67%)			33 (55.00%)
Grade 3	7 (4.33) (11.67%)	2 (3.67) (0.09%)	1 (2.00) (8.33%)	12.681	P<0.05	10 (16.67%)
Total of columns	26 (43.33%)	22 (36.67%)	12 (20.00%)			60
Necrosis						
No	24 (19.93) (92.31%)	19 (16.87) (86.36%)	3 (9.20) (25.00%)			46 (76.67%)
Yes	2 (6.07) (7.69%)	3 (5.13) (13.64%)	9 (2.80) (75.00%)	13.619	P<0.05	14 (23.33%)
Total of columns	26 (43.33%)	22 (36.67%)	12 (20.00%)			60
Mitotic count/HPF						
0	19 (14.30) (73.08%)	12 (12.10) (54.55%)	2 (6.60) (16.67%)			33 (55.00%)
1	4 (5.20) (15.38%)	6 (4.40) (27.27%)	2 (2.40) (16.66%)			12 (20.00%)
2	2 (3.03) (7.69%)	3 (2.57) (13.64%)	2 (1.40) (16.67%)			7 (11.67%)
>4	1 (3.47) (3.85%)	1 (2.93) (4.54%)	6 (1.60) (50.00%)	21.488	P<0.05	8 (13.33%)
Total of columns	26 (43.33%)	22 (36.67%)	12 (20.00%)			60

Low grade MCTs showed diffuse to moderate immunopositivity (Fig 3.7A) and high grade MCTs showed mild to nonspecific immunopositivity of VEGF (Fig 3.7B). High expression of VEGF was related to well and intermediately differentiated MCTs. The second method was used to evaluate the VEGF expression in angiogenesis in MCTs as described in previous study. Highly differentiated MCTs were low vascularized and the poorly differentiated MCTs were highly vascularized. High-grade canine cutaneous MCTs revealed highly vascularized areas and increased expression of VEGF around blood vessels as compared to low-grade MCTs (Fig 3.7A-B).

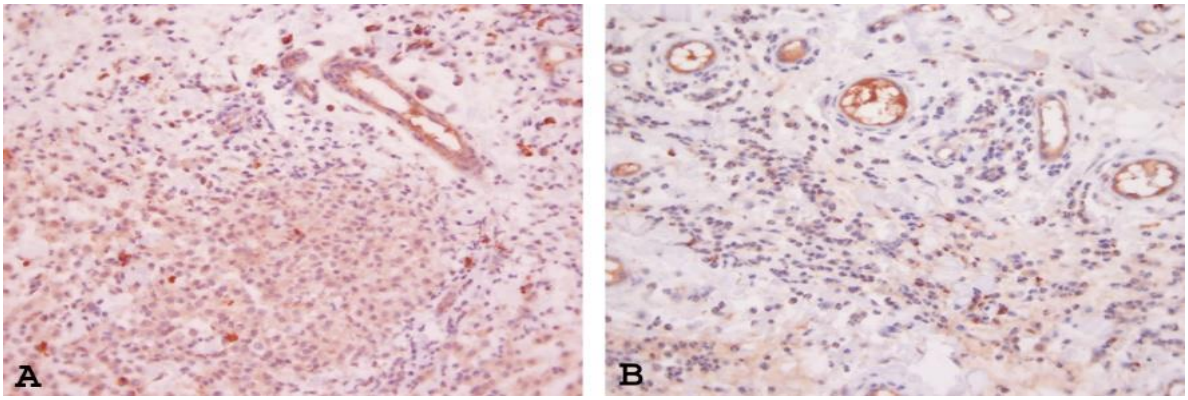

Fig 3.7: Evaluation of VEGF immunopositivity. (ABC-P, AEC chromogen, Mayer's Hematoxylin counterstain, 40x). A: Low-grade MCTs showed less vascularized areas and diffuse to moderate immunopositivity with VEGF. B: High-grade MCTs revealed highly vascularized areas and expression of VEGF was less intense.

3.3.6. Evaluation of TNF-α Immunopositivity

The immunopositivity of TNF-α was evaluated according to the intensity of stain. The intensity was graded as nonspecific positivity (-), mild positivity (+), moderate positivity (++), diffuse positivity (+++), according to the method described in previous study of VEGF expression. Thirty-three MCTs showed (+), 17 MCTs showed (++) and 10 MCTs showed (+++) immunopositivity with TNF-α. The relation between TNF-α immunopositivity and grades of MCTs, presence of necrosis and MC was investigated in this study. A significant correlation was found between TNF-α and grades of MCTs, presence of necrosis and MC (Table 3.7).

Table 3.7: TNF-α immunopositivity and other histopathological variables.

Grades of tumors	**TNF-α** +	**TNF-α** ++	**TNF-α** +++	Chi-square value	P-value	Total of rows
Grade 1	12 (9.35) (36.37%)	4 (4.82) (23.53%)	1 (2.83) (10.00%)			17 (28.33%)
Grade 2	20 (18.15) (60.60%)	10 (9.35) (58.82%)	3 (5.50) (30.00%)			33 (55.00%)
Grade 3	1 (5.50) (3.03%)	3 (2.83) (17.65%)	6 (1.67) (60.00%)	18.404	P<0.05	10 (16.67%)
Total of columns	33 (55.00%)	17 (28.33%)	10 (16.67%)			60
Necrosis						
No	30 (25.30) (90.91%)	15 (13.03) (82.24%)	1 (7.67) (10.00%)			46 (76.67%)
Yes	3 (7.70) (9.09%)	2 (3.97) (11.76%)	9 (2.33) (90.00%)	29.858	P<0.05	14 (23.33%)
Total of columns	33 (55.00%)	17 (28.33%)	10 (16.67%)			60
Mitotic count/HPF						
0	27 (18.15) (81.82%)	4 (9.35) (23.52%)	2 (5.50) (20.00%)			33 (55.00%)
1	3 (6.60) (9.09%)	8 (3.40) (47.05%)	1 (2.00) (10.00%)			12 (20.00%)
2	2 (3.85) (6.06%)	4 (1.98) (23.53%)	1 (1.17) (10.00%)			7 (11.67%)
>4	1 (4.40) (3.03%)	1 (2.27) (5.88%)	6 (1.33) (60.00%)	40.922	P<0.05	8 (13.33%)
Total of columns	33 (55.00%)	17 (28.33%)	10 (16.67%)			60

Well differentiated MCTs revealed diffused (nucleus and cytoplasmic) positivity (Fig 3.8A), intermediately differentiated MCTs also revealed diffused but some intermediately differentiated MCTs revealed moderate positivity (Fig 3.8B). Poorly differentiated MCTs revealed mild positivity and some poorly differentiated MCTs were not able to show any positivity (Fig 3.8C). Low-grade MCTs showed diffuse to moderate immunopositivity, whereas high-grade MCTs revealed mild to nonspecific immunopositivity of TNF-α.

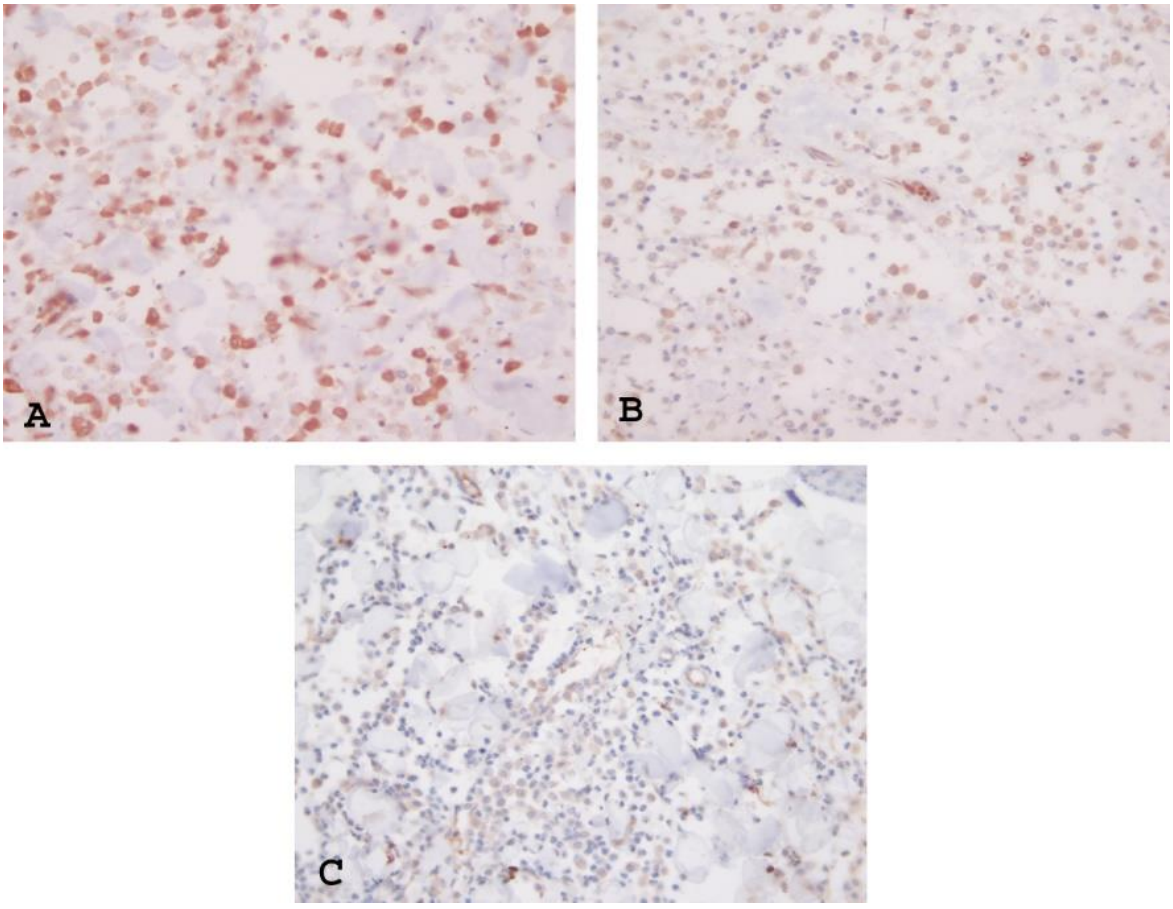

Fig 3.8: Evaluation of TNF-α immunopositivity. (ABC-P, AEC chromogen, Mayer's Hematoxylin counterstain, 40x). A: Well differentiated MCTs revealed diffused (nucleus and cytoplasmic) positivity. B: intermediately differentiated MCTs also revealed moderate positivity. C: Poorly differentiated MCTs revealed mild positivity and some poorly differentiated MCTs were not able to show any positivity.

3.3.7. Statistical Evaluation of All the Markers

The intensity of KIT positivity was also graded as nonspecific positivity (-), mild positivity (+), moderate positivity (++), diffuse positivity (+++), according to the method described in previous study of VEGF expression for the investigation of correlation of KIT with other markers. Ten MCTs showed (+), 32 MCTs showed (++) and 18 MCTs showed (+++) immunopositivity with TNF-α. A significant correlation was found between KIT and all other markers (Ki67, VEGF, OPN, Oct-3/4 and TNF-α). According to statistical results of all the markers, significant correlation was found between all the immunohistochemical markers used in current study. The detail of statistical values of all the immunohistochemical markers are given in Table 3.8.

Table 3.8: A significant correlation between all the immunohistochemical markers.

	+	++	+++	Chi-square value	P-value
KIT	10 (16.67%)	32 (53.33%)	18 (30.00%)	14.565	P<0.05
Ki67	28 (46.67%)	16 (26.66%)	16 (26.67%)		
VEGF	26 (43.33%)	22 (36.67%)	12 (20.00%)		
OPN	18 (30.00%)	17 (28.33%)	25 (41.67%)		
Oct-3/4	18 (30.00%)	16 (26.67%)	26 (43.33%)		
TNF-α	33 (55.00%)	17 (28.33%)	10 (16.67%)		

4. DISCUSSION

The abnormal or neoplastic transformation of mast cells leads to mast cell tumors in dogs. This disease is also called mastocytosis in humans. Mastocytosis is a hematological disease caused by the accumulation of abnormal mast cells in the tissues (Gümüşburun et al., 2019). MCTs in dogs are very common and account for 20% of all skin tumors in dogs (London and Seguin, 2003; Blackwood et al., 2012; Berlato et al., 2021). According to the location, there are three types of MCTs in dogs (cutaneous, subcutaneous, and extracutaneous) (Endicott et al., 2007; Marconato et al., 2008; Blackwood et al., 2012).

MCTs in dogs were reported at different ages, even in a 2-weeks-old dog (Davis et al., 1992a). In this study, the lowest age of the dog diagnosed with canine cutaneous MCT, was 3 years. Two dogs were diagnosed with cutaneous MCTs at the age of 3 years in this study. Different mean ages were reported in previous studies (Hottendorf and Nielsen, 1967; Strefezzi et al., 2003; Sabattini et al., 2021) but in this study mean age of dogs was 9.25 years for the development of canine cutaneous MCTs.

The development of MCTs has been reported in many different breeds of dogs (Hottendorf and Nielsen, 1967; Miller, 1995; McNiel et al., 2006; White et al., 2011). In this study 18 different breeds of dogs were presented including Golden Retrievers, Dogo Argentinos, Labradors, Pugs, French Bulldogs, Siberian Huskies, Cane Corso, Jack Russel Terrier, Dachshund, Samoyed, German Shepherd, American Pitbull Terrier, Cocker Spaniel, English Pointer, Terrier, American Bulldog, and Boxer. Less aggressive behavior of cutaneous MCTs in boxer and pug breeds and more aggressive behavior of cutaneous MCTs have been reported in Shar-peis breed (Miller, 1995; McNiel et al., 2006). In this study, same breeds also showed less aggressive behavior but Golden Retriever breed showed more aggressive behavior of cutaneous MCTs. This study was conducted in Türkiye and every country has its own breeds and this difference may be due to this. High incidence rate of cutaneous MCTs has also been reported in Golden Retriever and mixed breed dogs (Kiupel et al., 2011; Melo et al., 2021). In this study, 13 MCTs in golden

Retrievers and 8 MCTs in mixed breeds of dogs were identified. This study also showed the high incidence rate of cutaneous MCTs in Golden Retriever and mixed breed dogs.

Different sizes of cutaneous MCTs were diagnosed ranging from few mm to 20 cm in this study. Nodular rashes to diffuse swellings or hairless, raised, erythematous to highly variable tumors were identified. Poorly circumscribed lesions with ulcerated and pruritic surfaces, and rapidly growing behavior were observed in most high-grade canine cutaneous MCTs. Cut surface of canine cutaneous MCTs showed white or pink, sometimes with foci of hemorrhages in previous studies (Newman et al., 2007; Thompson et al., 2011) and same findings were also identified in the cut surface of cutaneous MCTs in this study.

There was no study to highlight either the male dogs have high incidence of MCTs or female dogs but in previous study total 33 dogs (17 females and 16 males) were diagnosed for MCTs. Even in the study of Kiupel 57 female and 38 male dogs were presented (Kiupel et al., 2011). The ratio of female dogs was high as compared to male dogs (Kiupel et al., 2011; Melo et al., 2021). In this study, the incidence rate of MCTs was also higher in female dogs as a total of 60 dogs (33 females and 27 males) were diagnosed with MCTs.

According to the 3-tier grading system (Patnaik et al., 1984), well-differentiated tumors were graded as grade 1, intermediately differentiated tumors were graded as grade 2, and poorly differentiated tumors were graded as grade 3 canine cutaneous MCTs in this study. Sabattini et al. (2015) also graded MCTs according to the Patnaik grading system, and 18 MCTs (13.1%) were identified as grade 1, 83 (61%) as grade 2, and 36 (26%) as grade 3 MCTs. In another study, 35 (34.0%) MCTs were graded as grade 1, 45 (43.7%) as grade 2 and 22(22.3%) were graded as grade 3 MCTs (Da Costa et al., 2007). Other study also stated 6(8.3%) MCTs as grade 1, 52 (75.4%) as a grade 2 and 11 (16.3%) as grade 3 MCTs in dogs (Fonseca-Alves et al., 2015). The results of this study were 14 grade 1, 30 grade 2 and 16 grade 3 canine cutaneous MCTs. If we compare the results of this study to the results of previous studies on the basis of 3-tier grading system, quite similar results were found.

The incidence rate of grade 2 MCTs was found to be high in both this study and previous studies. According to 2-tier system (Kiupel et al., 2011), different criteria were followed for the diagnosis and grading of canine cutaneous MCTs. The criteria were the presence of at least 7 mitotic figures, at least 3 multinucleated cells, at least 3 bizarre nuclei in 10 HPF or the presence of karyomegaly for the confirmation of high-grade MCTs. The absence of all these findings was the criteria for the diagnosis and grading of low-grade MCTs. In this study same criteria were followed for the diagnosis and grading of high-grade and low grade MCTs.

Mitotic figures, multinucleation, bizarre nuclei, and karyomegaly were reported in the high-grade MCTs of this study. Kiupel et al. (2011) stated that 90% cutaneous MCTs were low grade MCTs according to his 2-tier grading system because from total 95 MCTs 85 low grade and 10 high grade MCTs were diagnosed in that study. In another study, a total 53 dogs were diagnosed with 46 low and only 7 high grade MCTs. The percentage of low grade MCTs was almost 87%, and 13% of high-grade MCTs were diagnosed in that study (Vascellari et al., 2013). A different study also reported 28 low-grade and 19 high-grade MCTs and the percentage of low-grade was almost 60% and 40% for high-grade MCTs (Takeuchi et al., 2013). Seventy-one (86%) low-grade and 12 (14%) high-grade MCTs were reported in a previous study (Stefanello et al., 2015). In another study 56 (81%) low-grade and 13(19%) high-grade MCTs were diagnosed (Fonseca-Alves et al., 2015). The results of this study were 36 (60%) as a low grade and 24 (40%) high-grade canine cutaneous MCTs. The results of this study were also quite similar to the previous studies because the percentage of low grade MCTs was also high in this study. According to the Kiupel 90% of cutaneous MCTs were found to be low-grade MCTs (Kiupel et al., 2011) but according to the other studies (Vascellari et al., 2013; Takeuchi et al., 2013; Stefanello et al., 2015; Fonseca-Alves et al., 2015) and this study, the percentage of low-grade cutaneous MCTs can be different. On the basis of recent and old studies the percentage of low-grade cutaneous MCTs was 60-90% and 10-40% for high-grade cutaneous MCTs.

Both the 3-tier and 2-tier grading systems for the diagnosis of canine cutaneous MCTs have been reported in previous studies (Kiupel et al., 2011; Takeuchi et al., 2013; Vascellari et

al., 2013; Sabattini et al., 2015; Stefanello et al., 2015; Fonseca-Alves et al., 2015). According to the results of previous studies, all the grade 1 MCTs were graded as low-grade and all the grade 3 MCTs were graded as high-grade cutaneous MCTs. A high number of grade 2 MCTs were graded as low-grade canine cutaneous MCTs. The 60-80% grade 2 MCTs were graded as high-grade MCTs according to previous studies. It also has been reported in that studies that 2-tier grading system has higher inter-observer consistency as compared to 3-tier grading system of MCTs (Kiupel et al., 2011; Takeuchi et al., 2013; Vascellari et al., 2013; Sabattini et al., 2015; Stefanello et al., 2015; Fonseca-Alves et al., 2015). In this study, both 3-tier and 2-tier grading system were applied for the grading of canine cutaneous MCTs and quite similar results for the grades of MCTs were observed. As all the grade 1 MCTs were graded as low grade, all the grade 3 MCTs were graded as high grade and the high number of grade 2 MCTs were low grade canine cutaneous MCTs. The 2-tier grading system was easier to grade the canine cutaneous MCTs and the results of this study also support the higher inter-observer consistency of 2-tier grading system.

According to Patnaik's study, 36% of MCTs were diagnosed as grade 1, 43% were graded as grade 2 and 20% were graded as grade 3 canine cutaneous MCTs. The follow-up data from 1500 days revealed that 7% dogs in grade 1, 56% dogs of grade 2 and 94% dogs in grade 3 MCTs died due to MCT-associated disease. The percentage of dead dogs after 1500 days was 46%. The significant difference was found between the survivals of dogs and grades of MCTs (Patnaik et al., 1984). In the comparison of this study, 28.33% MCTs were graded as grade1, 55% were graded as grade 2, and 16.67% were graded as grade 3 canine cutaneous MCTs. According to the follow-up data of Patnaik's study, 1 dog from grade 1, 18 dogs from grade 2 and 9 dogs from grade 3 MCTs should have to die in 1500 days. The remaining dogs will be 16 (50%) with grade 1, 15 (47%) with grade 2 and 1(3%) with grade 3 MCTs. The statistical data of this study was also significant ($P<0.05$) after following the percentages of follow-up data of Patnaik's study (Patnaik et al., 1984).

According to Kiupel's study, 85 (89.47%) MCTs were diagnosed as low-grade and 10 (10.53%) were diagnosed as high-grade MCTs. The dogs with high grade MCTs showed a

shorter survival time as compared to the dogs with low-grade MCTs (Kiupel et al., 2011). According to the comparison of follow-up data of Kiupel's study with the current study, 70% MCTs were diagnosed as low-grade and 30% were diagnosed as high-grade MCTs, and the survival of the dog with high-grade MCTs will be less than the dogs having low grade MCTs. The follow-up data of 4 years was used in Kiupel's study, and it was revealed that not only MCTs can cause mortality in dogs but also some other tumors and diseases can also cause mortality in dogs, especially in the dogs with low-grade MCTs, which revealed mortality with other associated problems in dogs (Kiupel et al., 2011). The significant results with grading and survival time of dogs were evaluated in Kiupel's study with both 3-tier and 2-tier grading system, and the results of the current study also showed similarities after using the follow-up data of both Patnaik and Kiupel's studies.

Grade 3 and high-grade canine cutaneous MCTs showed less survival time, and grade 1, 2 and low grade showed longer survival time. The study of Kiupel's also reported that 2-tier grading system was easier to diagnose the MCTs because there was some ambiguity while using 3-tier grading system for the diagnosis of MCTs (Kiupel et al., 2011). It was also proposed that MCTs grading is good parameter for the determination of prognosis in dogs, but the correct use of chemotherapy needs determination of KIT expressions (Kiupel et al., 2011).

MC was also considered important for the determination of prognosis in dogs with cutaneous MCTs (Patnaik et al., 1984; Romansik et al., 2007). MC can vary from 0 to 20 per HPF and in one study grade 2 MCTs showed MC more than 5 and the median survival time was 5 months in these dogs. The grade 2 MCTs showing MC less than 5 showed median survival time of 70 months. In canine cutaneous MCTs, a high number of MC was associated with a shorter median survival time (Romansik et al., 2007). The results of the MC for the current study were also quite similar to the previous studies and the grade 2 MCTs showed mostly MC of more than 5. The association between the grades and MC was found to be significant ($P<0.05$) in the current study and these results are relevant to previous studies (Da Costa et al., 2007; Romansik et al., 2007).

It was thought that MCTs did not show so much recurrence even when neoplastic cells are present near the margins of the tumor but some researchers still believe that recurrence chances can be high in MCTs having no clean margins. The skin is not a good organ to facilitate the metastasis or recurrence of MCTs and that is the main reason for the lower chances of recurrence of MCTs in dogs. CD25 marker was used in a previous study to identify the margins of MCTs, and positive results were found in grade 1 MCTs (Meyer et al., 2012). The presence of inflammatory mast cells on the margins of MCTs is the main problem for the correct margin evaluation. CD25 was able to differentiate the neoplastic cells from the inflammatory mast cells in only grade 1 MCTs and when CD25 was tested on high grade MCTs, the results were not positive. It was thought that CD25 is not a good marker for the margin evaluation in canine cutaneous MCTs (Meyer et al., 2012). It was proposed that there is need of some new markers for an accurate margin evaluation. For an accurate margin evaluation, the data from the clinician is very important. If clinician made some incisions on the tumor or on the corner then he should mention it in the report. The clinician should mark the margins of MCTs before surgical removal and also mention the margins and planes of MCTs. For the tumors less than 4 cm in diameter or grade 1 tumors, a skin margin of 2 cm and one facial plane are enough (Simpson et al., 2004; Fulcher et al., 2006). A margin of 1 cm is enough for both grade 1 and grade 2 MCTs, as reported in another study (Schultheiss et al., 2011).

Four-mm-deep margins were also enough for the complete removal of MCTs (Schultheiss et al., 2011). Lateral margins and fascial planes are important parameters for the successful removal and control of tumors (Pratschke et al., 2013). Three cm of lateral margin and one fascial plane have been reported for the complete removal of high grade tumors (Donnelly et al., 2015). That previous studies have not reported the distance of histologic free margin. They also reported that 40% of high grade MCTs revealed recurrence even after complete surgical removal (Donnelly et al. 2015). While performing surgery, it is very difficult to know the grade of the tumor which is why there are chances of 20% recurrence, 80% metastasis, and death in cases of high grade MCTs.

There has been reported that in the dog having grade 3 MCT has chances of 35% to live for at least 2 years. This chance was increased up to 89% in the dogs having grade 2 MCTs. In the dogs having grade 1 MCTs have chance of 100% to live for 2 years (Donnelly et al., 2015). Clean surgical margins in grade 1 MCTs have no chances of recurrence mostly (Scarpa et al., 2012). For the grade 2 MCTs, the recurrence chances were 5-11% and the time period was 2-24 months described in two previous studies (Séguin et al., 2001; Weisse et al., 2002). Dirty/incomplete excision or margins of less than 2 mm have 6-30% chances of recurrence in low and intermediately differentiated MCTs (Abadie et al., 1999; Murphy et al., 2004; Séguin et al., 2006; Brocks et al., 2008). A correct and consistent method should be used for the margin evaluation of MCTs in dogs.

The percentage of clean margins (81.8%) and dirty margins (18.2%) has been reported in a previous study (Melo et al., 2021). In the current study the margins of 70% of MCTs were clean and 20% were dirty. The margins of 10% MCTs were not clear because the clinicians had not provided proper data about the margins of MCTs. According to the comparison of the current study with the previous study, the results of clean and dirty margins are almost similar. Different markers (OPN, Oct-3/4, VEGF, and TNF-α) were used in the current study for the margin evaluation, and the grade 1 and some low grade MCTs showed positive results for the neoplastic mast cells. In the grade 2, grade 3 and high-grade MCTs, the results were not clear, and it was also difficult to differentiate between inflammatory and neoplastic MCTs. It is suggested that these markers be tested again in future studies to confirm their significance in margin evaluation.

The prognostication of MCTs is very important for determining the accurate survival time of dogs. KIT plays an important role in the diagnosis, prognosis and determination of the survival time of dogs. Aberrant KIT expression has also been associated with the negative prognosis of MCTs in dogs especially cutaneous MCTs (Reguera et al., 2000; Kiupel et al., 2004; Preziosi et al., 2004; Da Costa et al., 2007). Three patterns of KIT expressions were identified and all the three grade tumors have chances of showing any of the patterns. In a previous study, 42.9% of MCTs showed pattern I, 43.9% showed pattern II and remaining

13.2% of MCTs showed pattern III of KIT expression (Kiupel et al., 2004). In another study, 46.6% of MCTs showed pattern I, 44.7% showed pattern II, and only 7.8% showed pattern III of KIT expression (Da Costa et al., 2007). MCTs with KIT patterns II and III were linked to a shorter survival duration and a higher prevalence of local recurrence. Such findings were frequently independent of c-kit exon 11 mutations. In a subsequent investigation of dogs treated with vinblastine and prednisolone, dogs with KIT pattern III MCT had considerably lower DFIs and survival durations than those with KIT pattern II (Kiupel et al., 2004). The relation of KIT immunopositivity with the other histopathological parameters (presence of necrosis and MC) has been reported in a previous study (Da Costa et al., 2007). In that study, the significant correlation was found between KIT expression (membranous and cytoplasmic) and the presence of necrosis and MC.

The results of KIT patterns in the current study are also similar to the previous studies and the correlation between KIT expression and the presence of necrosis and MC was also found to be significant ($P<0.05$) in the current study. The relationship between KIT immunoreactivity and survival endpoints was not investigated. Most subcutaneous MCTs do not have ITD mutations in exon 11, but like cutaneous MCTs, KIT cellular localization patterns are related to local recurrence and metastasis (Thompson et al., 2011). Dogs with non-perimembranous KIT labeled subcutaneous MCTs (patterns II and III) exhibited a high incidence of local recurrence (21/24; 88%) and metastasis (11/12; 92%). In predicting local recurrence (12/24; 50%) and metastases (7/12; 58%), the KIT pattern was more sensitive than MC >4 (Thompson et al., 2011).

The proliferation of different cells is an important factor in the assessment of the diagnosis and prognosis of canine cutaneous MCTs. Ki67, AgNOR, MC, PCNA, VEGF, p53, MDM2, serotonin and serotonin receptor, COX-2, cyclin D1, prostaglandin E2 expressions, plasma histamine concentrations, and, to a lesser extent, p21 expression have all been reported in canine MCTs (Bostock et al., 1989; Simoes et al., 1994; Abadie et al., 1999; Ginn et al., 2000; Ishiguro et al., 2003; Wu et al., 2004; Wu et al., 2006; Webster et al., 2007; Maglennon et al., 2008; Fröberg et al., 2009; Vascellari et al., 2013; Melo et al.,

2021). These cells are important for the estimation of survival time, metastasis and DFI in dogs. The Ki67 index was calculated and reported in 1999 (Abadie et al., 1999).

The other cell counts (AgNOR, PCNA and MC) have also been reported in different studies of MCTs in dogs (Bostock et al., 1989; Simoes et al., 1994; Webster et al., 2007; Maglennon et al., 2008; Vascellari et al., 2013). MC is the main process that has been performed in routine cases. The differences in the counts of all these cells are on the basis of counting, total areas, regions, view size, microscopes, and also differences in staining methods. It should be done with care so that duplication is not present in the results especially during the diagnostic process. These limitations should be considered important and the results of MC, Ki67 and Ki67 × AgNOR score are important for the diagnosing of MCTs in dogs (Simoes et al., 1994; Kravis et al., 1996; Abadie et al., 1999; Sakai et al., 2002; Séguin et al., 2006; Scase et al., 2006; Romansik et al., 2007; Webster et al., 2007; Ozaki et al., 2007; Maglennon et al., 2008; Vascellari et al., 2013; Berlato et al., 2015).

The proliferation and counting of MC, Ki67 and AgNOR × Ki67 score were also routinely reported in routine for the prognosis of canine MCTs (Séguin et al., 2006; Webster et al., 2007; Thompson et al., 2011; Smith et al., 2017). The process of PCNA counting is also a good method for the estimation of prognosis, but the problem is that there is no correct cut-off value for PCNA count. Due to this reason it is not used in the process of prognosis estimation (Abadie et al., 1999; Scase et al., 2006; Webster et al., 2007). All these markers have their own importance. Some of them are important for the diagnosis and prognostication of canine MCTs, and some of them cannot show the proper cut-off value for the diagnosis and prognosis of canine MCTs. MC, Ki67, AgNOR and Ki67+AgNOR are the main markers for the diagnosis and prognostication of canine cutaneous MCTs (Simoes et al., 1994; Kravis et al., 1996; Abadie et al., 1999; Sakai et al., 2002; Séguin et al., 2006; Scase et al., 2006; Romansik et al., 2007; Webster et al., 2007; Ozaki et al., 2007; Maglennon et al., 2008; Vascellari et al., 2013; Berlato et al., 2015). After the KIT expression. Ki67 is a fraction marker, AgNOR is a marker of proliferation rate, and MC is the marker of phase index.

These three categories of cells are important for the identification of phases of the cell cycle (Webster et al., 2007; Berlato et al., 2015). The growth fraction and its speed are the important factors in determining the proliferation of cells, and no single marker can give accurate results. The dogs with the highest expression of Ki67 had a shorter survival time. The higher expression was more than 93 Ki67 positive cells out of 1000 cells. Twenty-nine grade 2 MCTs showed a Ki67 positivity score less than 93 and a higher survival time of 12 months. Ten dogs showing a Ki67 score more than 93 died before 12 months (Abadie et al., 1999). In another study, MCTs revealed positivity for Ki67, and the median survival time was 395 days for those dogs the 1-, 2-, and 3-year probabilities of survival were 0.43, 0.21, and 0.21 Ki67 scores, respectively (Maglennon et al., 2008). Ki67 positivity revealed survival probabilities of 0.92, 0.86, and 0.77 in another study. The median survival time was not calculated in that study (Bostock et al., 1989).

The survival probabilities were also evaluated and these were 0.95 and 0.92 for low-and-high grade MCTs, respectively (Vascellari et al., 2013). Five out of 13 dogs died due to MCT related diseases, and the Ki67 index was more than 10.6. The survival probabilities were also calculated and the values were 0.92 and 0.77. The survival time was 6 months and 12 moths, respectively for the low and high grade MCTs (Vascellari et al., 2013). The Ki67 score more than 23 was considered significant in high grade MCTs and less than 23 was related to low grade MCTs in most studies (Da Costa et al., 2007; Webster et al., 2007). The MCTs showing a Ki67 score more than 23 showed less survival time, while the MCTs showing a Ki67 score less than 23 showed long survival time in those studies. The correlation between Ki67 and KIT immunopositivity has been reported in a previous study (Da Costa et al., 2007). The correlation with other pathological variables (presence of necrosis and MC) has also been reported in that study. The results of Ki67 immunopositivity were also found to be quite similar with the previous studies and the correlation between Ki67 and other pathological variables (presence of necrosis and MC) was also significant ($P<0.05$) in current study.

The combined score of AgNOR × Ki67 was also considered important, and 56 dogs were used in a study, of which 8 revealed grade 1 MCTs. Forty-one dogs revealed grade 2 and seven dogs revealed grade 3 of MCTs. Fifteen dogs from the grade 2 and 3 dogs from the grade 3 MCTs revealed more than 54 combined score of AgNOR × Ki67 (Webster et al., 2007). The dogs revealing a combined score of AgNOR × Ki67 above 54 died before 12 months and other dogs showing this combined score less than 54 survived for 2 years (Webster et al., 2007). The combined score of AgNOR × Ki67 is really important for the prognosis and therapeutic purpose especially in the canine MCTs. The dogs having combined score of AgNOR × Ki67 above 54 but do not have internal tandem duplication (ITD) mutations in the exon 11 revealed good response of prednisolone/vinblastine combination. Tyrosine kinase inhibitor treatment was not impressive in these dogs (Webster et al., 2007).

There are many other molecular markers that were tested for the evaluation of microenvironment of canine MCTs and some other new molecular markers (VEGF, OPN, Oct-3/4, TNF-α were used in current study to evaluate the microenvironment and margin evaluation of canine cutaneous MCTs. The expression of VEGF has also been reported in some previous studies of canine MCTs (Rebuzzi et al., 2007; Patruno et al., 2009; Melo et al., 2021). There are different findings about the VEGF expression in canine MCTs. In one study it was stated that VEGF is not a good prognostic marker for the canine MCTs (Rebuzzi et al., 2007), but in other two studies, it was stated that VEGF can be used as a prognostic marker for the canine MCTs (Patruno et al., 2009; Melo et al., 2021). The level of VEGF in serum, platelet-poor plasma, cytosolic VEGF concentration, plasma activated platelet rich, microvascular density, and mast cell density were evaluated by ELIZA and immunohistochemistry in canine MCTs (Patruno et al., 2009). The grade 3 canine MCTs showed a higher level of VEGF as compared to grade 1 and 2 MCTs. The grade 3 MCTs showed higher vascularization as compared to other two grades of MCTs. It was suggested that mast cells secrete VEGF, which can be a good angiogenetic marker for MCT diagnosis and prognostication (Patruno et al., 2009).

In another study the correlation of VEGF with prognostication factors, histological grading, and c-kit was not found to be significant. It was only significant with the metastasis in that study (Melo et al., 2021). The results of VEGF expressions of current study were quite similar with the study of Paturno et al. (2009). But it was a little bit different from the study by Melo et al. (2021) because the correlation of VEGF expression was found to be significant with grades of MCTs, presence of necrosis, MC and KIT. According to the results of the current study, it can be suggested that VEGF might be included in the list of prognostic markers of canine cutaneous MCTs. Two different theories about the Oct-3/4 expression has also been found in previous studies of canine MCTs (Vargas et al., 2015; Meesuwan et al., 2021). In one study, only 28 canine MCTs were used for the evaluation of Oct-3/4 immunopositivity and 21 MCTs showed cytoplasmic and nuclear immunopositivity, while 6 MCTs showed only cytoplasmic immunopositivity and one MCT was not able to show any immunopositivity (Vargas et al., 2015).

The correlation between Oct-3/4, grades of tumor, mortality rate, and survival rate were evaluated in that study, and they have not found significant results. The expression of Oct-3/4 was also investigated at the protein and mRNA level in other studies but the significant results were not found might be due to the presence of pseudogenes and different isoforms of this protein (De Jong and Looijenga, 2006; Moulay et al., 2013). Oct-3/4 has also been reported as a good biological, prognostic, and progression marker in bladder cancer (Chang et al., 2008). The role of Oct-3/4 in the migration and invasion of glioblastoma cells has also been reported (Kobayashi et al., 2012). Oct-3/4 contributes to promoting the process of angiogenesis by producing VEGF during the development of a tumor (Takahashi et al., 2015).

According to the results of these studies it is clear that Oct-3/4 can produce VEGF, and VEGF is a good prognostic marker in the canine MCTs. The positive relationship between VEGF and Oct-3/4 is found and it can be suggested that Oct-3/4 might have importance in the diagnosis and prognostication of canine cutaneous MCTs. In another study the expression of Sox-2 and Oct-3/4 was evaluated in canine cutaneous MCTs. The expression

level of these two markers was evaluated at the protein and mRNA levels with the help of immunohistochemistry and RT-PCR. The correlation between the Oct-3/4 expression and grades and breeds was evaluated and significant positive results were found in that study (Meesuwan et al., 2021). It was suggested that Oct-3/4 might be a good marker for the diagnosis and prognostication of canine MCTs. Previous studies were performed on a smaller number of MCT samples. The results of the current study were also significant because a significant correlation was found between Oct-3/4, grades of MCTs, presence of necrosis, and MC. The expression of Oct-3/4 showed results that were similar to Vagas et al. (2015). According to the results of Oct-3/4 expression in the current study, it can be suggested that Oct-3/4 might be a good marker for the diagnosis and prognosis of canine cutaneous MCTs.

OPN was first time used in the current study of canine cutaneous MCTs. The results of one study stated that OPN is a mediator of mast cells and enhances the response of mast cells against antigens. It can also play an important role in mast cell related pathological diseases (Nagasaka et al., 2008). In an experimental study, a reduction in the growth of primary tumors and metastasis was reported due to the downregulation of OPN (Wu et al., 2000). In another experimental study, the role of OPN was explained in the experimental metastasis. It was stated that the OPN expression was able to convert the benign tumor cells to the complete metastatic form (Barraclough et al., 1998). The association of increased OPN expression with metastatic phenotypes has been reported, and these metastatic phenotypes were responsible for the selection and expression of different kinds of breast cancer cells in humans (Urquidi et al., 2002; Kang et al., 2003).

A model study of squamous cell carcinoma was conducted in mice, and the tumors revealed more malignancy in the rats with deficient OPN. The metastasis of tumor in lungs were also numerous in the rats with deficient OPN (Crawford et al., 1998). In that study, the host cells expressed the really important role of OPN in tumorigenesis. The development of primary tumors was quite similar in all the rats, with or without deficient OPN. These tumors did not actually express the proper effect of OPN on the metastasis (Feng and Rittling, 2000;

Chen and Rittling, 2003). In another experimental study, melanoma cells were used, and they revealed weak expression of OPN and lower number of metastases in the mice with deficient OPN (Nemoto et al., 2001). According to the results of these experimental studies, it is clear that OPN can play an important role in tumor development and that it can be affected by different parameters. These parameters mayinclude the type of tumor and the experimental system. It can also reflect the important activity of tumor microenvironment for the determination of OPN effect. It has been suggested that the different cells in the microenvironment of tumors may produce OPN that has the ability to play different functions. These cells of the tumor microenvironment include immune cells, remodeling blood vessels, bone cells, or the tumor cells themselves.

It was suggested in a study that OPN expression is related to the good prognosis of medullary thyroid cancer (Ferreira et al., 2016). The expression of OPN in canine MCTs was quite interesting, and fully differentiated MCTs showed diffuse positivity, while the poorly differentiated MCTs showed very low/negative positivity. The positive mast cells were also found on the periphery of less differentiated MCTs, which can be important for the margin evaluation of MCTs. These reactive positive mast cells were away from the malignant cells, and they can also play an important role in the differentiation of MCTs. The statistical correlation was evaluated between OPN expression, grades of tumors, presence of necrosis and MC. The significant correlation was between OPN and different pathological variables. According to these results, it can be suggested that OPN might be a good marker for the differentiation, diagnosis, prognostication and margin evaluation of canine cutaneous MCTs.

TNF-α expression was also evaluated for the first time in the current study of canine cutaneous MCTs. The production of TNF-α by the mast cells has been reported in previous studies (Gordon and Galli, 1990; Gordon and Galli, 1991). Dual role of TNF-α in the tumors has been reported in previous studies (van Horssen et al., 2006; Cruceriu et al., 2020). TNF-α can play antitumoral role together with melphalan and also play important role in the progression of tumors. The important role of TNF-α in the skin, hepatic and

gastrointestinal carcinogenesis has been reported in previous study (Kovacevic et al., 2008). The role of TNF-α has been found in the gastrointestinal carcinogenesis and it also has been expressed in the biopsies of colorectal cancer and ulcerative colitis (Popivanova et al., 2008). In the current studies, TNF-α expression was quite interesting and fully differentiated MCTs showed specific positivity and less differentiated MCTs showed low/negative positivity. The reactive positive mast cells were also found at the peripheries of less differentiated MCTs which can be an important factor in the differentiation of different grades and the margin evaluation of canine cutaneous MCTs. The significant correlation was found between TNF-α, grades of MCTs, presence of necrosis and MC. The statistical results can suggest that TNF-α might be the good marker for the differentiation, diagnosis, prognostication and margin evaluation of canine cutaneous MCTs.

The correlation between all the markers was also found to be significant ($P<0.05$) in the current study. Significant correlation between KIT, Ki67, AgNOR and VEGF were also reported in previous studies (Webster et al., 2007; Da Costa et al., 2007; Fonseca-Alves et al., 2015). The KIT, Ki67, VEGF expressions of current study were considered specific markers for the differentiation, diagnosis and prognosis of canine cutaneous MCTs because the results of these markers were quite similar with the previous studies. The proper data for the expressions of other markers (OPN, Oct-3/4 and TNF-α) was not found in previous studies. The expression of some other markers (serotonin and its receptor 5-HT1A (5-hydroxy tryptamine), Mdm2 and P53) have also been reported in previous studies (Wu et al., 2006; Fröberg et al., 2009). The expressions of serotonin, 5-HT and P53 were found positive in well differentiated MCTs and negative expressions were found in less differentiated tumors. The immunohistochemical expression of OPN, Oct-3/4 and TNF-α of current study might be similar with these previous studies.

The expression of these markers was found less with the increase of differentiation of canine MCTs. The reactive positive mast cells in the periphery of less differentiated MCTs were found positive with all three markers, and that can be a positive sign for the differentiation and margin evaluation of canine cutaneous MCTs. The correlation of these

markers with the grades of MCTs, the presence of necrosis, MC, and other markers was found to be significant and that can be a positive point for the suggestion of these markers. According to these results it can be suggested that KIT, Ki67, VEGF, OPN, Oct-3/4 and TNF-α might have important role in the differentiation, diagnosis, prognostication and margin evaluation of canine cutaneous MCTs. The presence of different markers has been reported in the microenvironment of canine MCTs (Bostock et al., 1989; Simoes et al., 1994; Kravis et al., 1996; Abadie et al., 1999; Ginn et al., 2000; Ishiguro et al., 2003; Wu et al., 2004; Wu et al., 2006; Webster et al., 2007; Maglennon et al., 2008; Vascellari et al., 2013; Melo et al., 2021). The current study evaluated some previous (KIT, Ki67 and VEGF) and some new (OPN, Oct-3/4 and TNF-α) microenvironmental markers for the differentiation, diagnosis, prognostication and margin evaluation of canine cutaneous MCTs.

There are some deficiencies in the current study including the cytological examination, follow-up data after the surgical removal of MCTs and the PCR evaluation for the confirmation of the c-kit mutation. In future research, a higher number of samples and all these parameters should be considered for more accurate results in the evaluation of microenvironmental markers. The purpose of this study was to evaluate the microenvironmental markers in the canine cutaneous MCTs. It also included the diagnosis (comparison of the 3-tier and 2-tier grading systems), differentiation, margin evaluation, and prognostication of canine cutaneous MCTs with the help of histopathological and immunohistochemical evaluation.

5. Conclusions and Recommendations for the Accurate Diagnosis and Prognostication of Canine Cutaneous MCTs

After evaluating the microenvironmental markers in canine cutaneous MCTs by histopathological and immunohistochemical methods, we made a result that before the conclusions of this study there should be some recommendations to the clinicians, oncologists and the researchers for the accurate diagnosis and prognostication of canine cutaneous MCTs. FNA method for the initial diagnosis of cutaneous MCTs should be considered as it can diagnose 80-90 % of cases. A two-tier grading system might be used in FNA method for diagnosis. Loss of granularity, mitotic figures, multinucleation, pleomorphism and anisokaryosis (>50%) are the main identifying points for the high grade cutaneous MCTs with FNA method. Most tumors (almost 1/3) will be diagnosed as high grade MCTs with the FNA methods. So histopathological evaluation should be recommended always for the prognostic evaluation. An excisional biopsy should be performed after the evaluation of FNA. The first surgery should be done properly with the thinking of first cure of the MCTs. The tumor of less than 4 cm diameter should be excised with 1 cm lateral margins and 4mm deep margins. After the confirmation of more aggressive MCTs with the FNA method, lateral margin of 3 cm and deep margin of one fascial plane should be excised. Non resectable tumors should be tested with incisional biopsy and grading should be done for further treatment, but this method is not very reliable.

The excisional biopsy should be performed carefully, and the surgical margins should be inked properly for the histopathological evaluation of cutaneous MCTs. There is no need of margin evaluation for the well circumscribed MCTs that have wide surgical margins. A complete margin evaluation should be recommended in the MCTs showing narrow margins. For the complete margin evaluation short and long axis measurement should be done. The distance between the tumor and the normal tissue in the deep and lateral margins should be measured properly. Enlarged lymph nodes should also be tested with the FNA method. The tumor needs no further staging if it is low-grade tumor and does not have

metastasis in the lymph nodes. Some dogs may show the clinical signs of vomiting and melena, then ultrasound should be done. Full staging and systemic therapy should be considered in the high-grade MCTs that have metastasis in the lymph nodes, and the lymph nodes should also be removed during the surgery and checked with the histopathology method. FNA of internal organs (liver, spleen and bone marrow) should also be considered in dogs showing internal clinical issues with ultrasound. During the selection of additional therapy, there should be considered 3 parameters in the mind that indication of local, systemic or inclusion of tyrosine kinase receptors in systemic therapy.

The decision for the consideration of additional local therapy should be made after a complete evaluation of the margins, histological grading, and proliferation markers. Local additional therapy is not required in the MCTs with clean margins. When tumor is low-grade but the margins are not clean, additional local therapy should be recommended after the evaluation of proliferation markers (KIT, Ki67, and VEGF). If there is less expression of proliferation markers in the tumor with dirty margins then there will be no need for additional local therapy. If the low-grade or some high-grade MCTs have dirty margins and are also showing higher expressions of proliferation markers, then additional local therapy should be considered. For the high-grade MCTs showing incomplete or narrow margins, local therapy should also be considered.

For the selection of systemic therapy, evaluation of histological grades, proliferation markers, PCR for the confirmation of the c-kit mutation (exon 11 or others), and the KIT expressions with immunohistochemistry should be done. For the high-grade cutaneous MCTs showing metastasis and aggressive behavior, systemic therapy should be considered. Systemic therapy should not be considered in low-grade MCTs showing no metastasis, less expression of proliferation markers (Ki67, VEGF), no c-kit mutation and also showing KIT pattern 1. Systemic therapy should be considered for the low-grade MCTs that have no metastasis but show high expression of proliferation markers. The cutaneous MCTs showing metastasis should be considered for systemic therapy. In the low grade MCTs

which are not showing metastasis but c-kit mutation is positive on exon 11 and also showing KIT pattern of II or III, the systemic therapy should be considered.

For the selection of tyrosine kinase inhibitors as a systemic therapy for the treatment of canine cutaneous MCTs, PCR for the detection of c-kit mutation (on exon 11 or others) and expressions of KIT patterns (II or III) should be evaluated. Tyrosine kinase inhibitors therapy should be considered in MCTs showing a c-kit mutation on exon 11 or 8 and also showing expression of KIT patterns II or III. The tyrosine kinase inhibitors therapy should not be considered in the MCTs showing no c-kit mutation and showing expression of KIT pattern I.

A mean age of 9.25 years has been found for the development of canine cutaneous MCTs in dogs. The Golden Retriever and mixed breeds of dogs showed a high incidence of cutaneous MCTs. The comparison of 3-tier and 2-tier grading system was done and the 2-tier grading system and it was found that 2-tier grading system has more inter-observer consistency for the diagnosis and prognostication of canine cutaneous MCTs. Margin evaluation plays important role in the diagnosis and prognostication of canine cutaneous MCTs. The markers (OPN, Oct-3/4 and TNF-α) might play an important role in the margin evaluation. The correlation between the markers (KIT, Ki67, VEGF, OPN, Oct-3/4 and TNF-α) and other histopathological variables (presence of necrosis and MC) was considered significant. The correlation between all the markers was also significant that showed the importance of the proliferation of markers in the diagnosis and prognostication of canine cutaneous MCTs. The KIT, Ki67 and VEGF were the reliable markers for the differentiation, diagnosis, prognostication and selection of chemotherapy in canine cutaneous MCTs. After evaluating the immunohistochemical and statistical results, it can also be suggested that other markers (OPN, Oct-3/4, and TNF-α) might be good microenvironmental markers for the differentiation, diagnosis, and prognostication of canine cutaneous MCTs.

All the methods evaluated in the current study have their own importance. There are always exceptions to everything, even in the results of different studies. There should be constant communication between clinicians, oncologists and researchers for accurate diagnosis, and research purposes. Diagnostic and treatment approaches are always selected after the proper histopathological and molecular evaluation of canine cutaneous MCTs. Clinicians, oncologists and researchers should work in collaboration for the proper diagnosis and treatment of canine cutaneous MCTs.

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