Novel Prognostic and Predictive Markers for Canine Mast Cell Tumours

by

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ABSTRACT

NOVEL PROGNOSTIC AND PREDICTIVE MARKERS FOR CANINE MAST CELL TUMOURS

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Mast cell tumours (MCTs) are the most common skin tumour of the dog, representing approximately 21% of all cutaneous tumours. Accurately predicting behaviour is critical in directing patient therapy, especially in canine MCTs as they range from benign to a fatal systemic disease. Grading is useful for prognosis, but it cannot predict the behaviour of each MCT. We hypothesized that biomarker staining in tumour tissues will correlate with patient outcome. A clinically annotated tissue microarray of primary, recurrent, and metastatic cutaneous canine MCTs (with and without adjunctive treatment) was created and high-throughput immunohistochemical staining profiling of 244 tumours from 189 dogs was performed. Total staining of KIT, a receptor tyrosine kinase that is an important driver of MCTs, was not prognostic. Mast cell tryptase levels were found to be prognostic in low-grade MCTs, with low tryptase-expressing tumours having a decreased time to recurrence and/or metastasis compared to high-tryptase expressing tumours. Two other proteins involved in protein degradation pathways were also investigated: c-CBL, an E3 ubiquitin ligase that also functions as an adaptor protein to regulate signaling pathways, and beclin-1, an autophagy protein. High c-CBL expressing tumours had a decreased MCT-related survival time in primary, adjunctive therapy treated, subcutaneous MCTs. Beclin-1 staining level was a strong predictive biomarker for cutaneous MCTs. High beclin-1 expressing tumours showed poor response to adjunctive treatment compared to low beclin-1 expressing tumours, especially for high-grade or high mitotic count tumours. These
findings will hopefully improve our ability to prognosticate MCTs and help decide whether to pursue adjunctive treatment. Importantly, this is the first evidence that autophagy inhibitors may be useful in improving response to treatment for dogs with high-grade MCTs.
Acknowledgements

I owe a big debt of gratitude to the many people who have helped me in the completion of this thesis and my program.

Rob, you have made my time here a true pleasure. Your vast knowledge and teaching have helped me develop as a pathologist, while your caring and compassion have warmed my heart. Thank you for everything you have taught me, thank you for all the conversations about pathology and life in general, and thank you for your unending support. Brenda, thank you for making this thesis possible. You allowed me to work independently, while giving me excellent advice and guidance for my project (and research/academia/life in general) along the way. I have thoroughly enjoyed working on this project with you and our conversations, and for that I truly thank you.

I would like to thank Geoff Wood for not only being a part of my thesis committee and giving me many helpful suggestions, but also allowing me to work closely with his laboratory to help conduct my experiments. A huge thank you also goes to Courtney Schott, who helped me tremendously by training me to make a TMA and giving me other advice. Thank you to Tami Sauder for always happily cutting sections for me. Thank you to Mary Ellen Clark for her help in the lab, and to Karolina Skowronski with her help in collecting outcome data.

A big thank you to the anatomic and clinical pathologists in Pathobiology. I am still amazed that it is possible to assemble a team of pathologists with such high-quality pathology skills and knowledge while also being compassionate and kindhearted. I have learned so much from each of you. Thank you to the always helpful staff in Pathobiology. Thank you also to the pathologists and staff at the Animal Health Laboratory. Thank you to Susan Lapos for her help with the immunohistochemistry for this project, as well as the rest of the AHL histopathology team for their smiles, their friendships and their help for my pathology cases. Thank you to the entire post mortem room crew for their immense and invaluable help, especially when things got busy.

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Thank you to the Ontario Veterinary College Pet Trust Organization for funding this study and thank you to the University of Guelph and OVC Awards that provided me with financial support. Thank you to the staff at the veterinary clinics who provided me with the outcome data. I am grateful to all the patients and owners who were involved in this study, and I hope this work leads to improved treatments.

There are two people without whom, I would not have been able to accomplish all that I have during my program. I am extremely grateful and fortunate to have such an extremely supportive husband, Boris Lee, and extremely supportive mother, Dagmar Knight. I cannot express in words my thanks to both of you for supporting me every step of the way. Mom, thank you for everything you have done for me, and for all your help with the boys. I am so lucky to have the best mom in the world. Boris, you have done so much for me, in so many ways. Thank you for being not only an incredible partner, but also an amazing father. I love you.

Finally, I would like to dedicate this thesis to my two boys, Alex and Jakob. You two have opened my mind and heart to a whole new world that I never knew existed. Thank you for the deep love and pure joy that you have brought into my life. I love you both so much.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNOR</td>
<td>Argyrophilic nucleolar organizer region</td>
</tr>
<tr>
<td>AHL</td>
<td>Animal Health Laboratory</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>AT</td>
<td>Adjunctive therapy</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>C-CBL</td>
<td>C-casitas B-lineage lymphoma</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>Macrophages colony stimulating-factor receptor</td>
</tr>
<tr>
<td>CTMC</td>
<td>Connective tissue mast cell</td>
</tr>
<tr>
<td>DAB</td>
<td>3-3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DFI</td>
<td>Disease-free interval</td>
</tr>
<tr>
<td>DIH</td>
<td>Digital image hub</td>
</tr>
<tr>
<td>ECF-A</td>
<td>Eosinophilic chemotactic factor-A</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibroblast activating protein</td>
</tr>
<tr>
<td>FceRI</td>
<td>High-affinity receptor for immunoglobulin E</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>Flt3</td>
<td>FLT cytokine receptor</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LC3</td>
<td>Light chain 3</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTB4</td>
<td>Leukotriene B4</td>
</tr>
<tr>
<td>LTC4</td>
<td>Leukotriene C4</td>
</tr>
<tr>
<td>MAdCAM-1</td>
<td>Mucosal addressin cellular adhesion molecule-1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC</td>
<td>Mitotic count</td>
</tr>
<tr>
<td>MCc</td>
<td>Mast cell with chymase expression</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MCps</td>
<td>Mast cell progenitor cells</td>
</tr>
<tr>
<td>MCt</td>
<td>Mast cell with tryptase expression</td>
</tr>
<tr>
<td>MCT</td>
<td>Mast cell tumour</td>
</tr>
<tr>
<td>MCtc</td>
<td>Mast cell with tryptase and chymase expression</td>
</tr>
<tr>
<td>MIP-α</td>
<td>Monocyte inflammatory peptide-α</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal mast cell</td>
</tr>
<tr>
<td>MST</td>
<td>Median survival time</td>
</tr>
<tr>
<td>NCF</td>
<td>Neutrophil chemotactic factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>OVC</td>
<td>Ontario Veterinary College</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>Platelet-derived growth factor receptor beta</td>
</tr>
<tr>
<td>PGD$_2$</td>
<td>Prostaglandin D$_2$</td>
</tr>
<tr>
<td>PI3</td>
<td>Phosphatidylinositol 3</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placenta growth factor</td>
</tr>
<tr>
<td>RT</td>
<td>Radiation therapy</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>ST</td>
<td>Survival time</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
</tbody>
</table>
Declaration of Work Performed

All work in these chapters was done by me, with the exception of sectioning of slides for immunohistochemistry, which was done by Tami Sauder, Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, and the automated immunohistochemistry which was done by Susan Lapos at the Animal Health Laboratory, University of Guelph, Guelph, Ontario. In addition, some of the outcome data was retrieved by Karolina Skowronski at the Institute for Comparative Cancer Investigation at the Ontario Veterinary College, University of Guelph, Guelph, Ontario.
Chapter 1: Literature Review

Mast cell biology

Mast cells were first described in 1878 by Paul Ehrlich during his doctoral thesis presentation (Crivellato et al., 2003). Believing that these granular cells served a nutritional function, he coined the term “Mastzellen”, as the German word “Mast” implies a “fattening” or “suckling” function (Beaven, 2009). Since that time, studies have revealed a wide and diverse range of functions for these cells, with mast cells playing important roles in both immunological and non-immunological settings (Crivellato et al., 2015; Galli et al., 2005). In humans, mast cells are strategically located at the interface between the host and the environment, such as the skin, and the mucosae of the respiratory, gastrointestinal, and genitourinary tracts, as well as around blood vessels and nerves (Abraham and Malaviya, 1997). They are present in large numbers, which is highlighted by the fact that their combined mass is estimated to be equal to that of the spleen (Caughey, 1991). Canine mast cells are also widely distributed with a localization pattern that closely follows that of humans (Table 1.1).

Table 1.1 Location of canine mast cells.

<table>
<thead>
<tr>
<th>Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>(Becker et al., 1985; Kube et al., 1998)</td>
</tr>
<tr>
<td>Adipose tissue of the subcutis</td>
<td>(Kube et al., 1998)</td>
</tr>
<tr>
<td>Airways</td>
<td>(Kube et al., 1998; Peeters et al., 2005; Su et al., 1993)</td>
</tr>
<tr>
<td>Small intestine</td>
<td>(German et al., 1999; Kube et al., 1998)</td>
</tr>
<tr>
<td>Large intestine</td>
<td>(German et al., 1999)</td>
</tr>
<tr>
<td>Stomach</td>
<td>(Kube et al., 1998; Soll et al., 1988)</td>
</tr>
<tr>
<td>Liver</td>
<td>(Morimoto et al., 1998; Yamamoto, 2000)</td>
</tr>
<tr>
<td>Uterus</td>
<td>(Kube et al., 1998)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>(Kube et al., 1998)</td>
</tr>
<tr>
<td>Heart</td>
<td>(Frangogiannis et al., 1998)</td>
</tr>
<tr>
<td>Brain</td>
<td>(Matsumoto et al., 2001)</td>
</tr>
<tr>
<td>Tunica albuginea of testes</td>
<td>(Anton et al., 1998)</td>
</tr>
<tr>
<td>Bone marrow (rare)</td>
<td>(Bookbinder et al., 1992)</td>
</tr>
</tbody>
</table>
Mast cell development

In humans, mice, and dogs, mast cells are derived from CD34+ hematopoietic progenitor cells in the bone marrow (Chen et al., 2005; Kirshenbaum et al., 1991; Lin et al., 2006). These cells leave the bone marrow as morphologically unidentifiable progenitors and travel to most organs and tissues of the body where they mature in situ into phenotypically identifiable mast cells (Hatanaka et al., 1979; Kitamura and Fujita, 1989). Additionally, white adipose tissue is also a site of functional mast cell precursors in mice (Poglio et al., 2010). The developmental pathway of mast cells in relation to other myeloid-derived cells remains somewhat controversial. One study described a progenitor cell that could give rise only to mast cells or basophils (Arinobu et al., 2005), cells that share many similar functions with mast cells (Crivellato et al., 2011). Multiple other studies suggest that basophils share a common progenitor with eosinophils, while mast cells develop from a lineage independent from granulocytes (Chen et al., 2005; Denburg et al., 1985; Franco et al., 2010).

The mast cell progenitor cells (MCps) found in the periphery are generally defined by their expression of the high-affinity receptor for immunoglobulin E (FceRI) and the stem cell factor (SCF) receptor KIT, as well as having the ability to form mast cell colonies in culture (Gurish and Austen, 2012). Notably, the most important factor in mast cell development and proliferation is the glycoprotein SCF (Kitamura and Go, 1979; Yee et al., 1994). This was first suggested in early studies with mice with mutations in either the W (c-KIT) locus (encoding KIT), or the Sl locus (encoding SCF) (Kitamura and Go, 1979; Kitamura et al., 1978). Partial loss-of-function mutations in either locus resulted in profound deficiencies in the number of tissue mast cells, in addition to hypopigmentation and sterility (Kitamura and Go, 1979; Kitamura et al., 1978), while basophil numbers were not significantly different between wild type and mutant W mice (Jacob et
Further studies *in vitro* demonstrated the potent proliferative effects of SCF on MCps (Sawai et al., 1999), as well as anti-apoptotic effects (Moller et al., 2005).

Stem cell factor is widely expressed during embryogenesis and in the adult. Stem cell factor can exist as a membrane-anchored or soluble isoform, depending on alternative RNA splicing and subsequent proteolytic cleavage (Huang et al., 1992). The differential expression of the two isoforms can vary widely between different tissues (Huang et al., 1992), and both isoforms can be produced in the bone marrow by human marrow stromal fibroblasts (Linenberger et al., 1995), as well as many other cells, including murine fibroblasts (Dvorak et al., 1994; Flanagan and Leder, 1990), human and rat hepatic stellate cells (Gaca et al., 1999), human epithelial cells (Wen et al., 1996), human bronchial smooth muscle cells (Kassel et al., 1999), human endothelial cells (Aye et al., 1992), human keratinocytes (Hamann et al., 1995), human and canine mast cells (de Paulis et al., 1999; Noli and Miolo, 2001), human breast carcinoma and small cell lung carcinoma cell lines (Turner et al., 1992), and canine mast cell tumours (Amagai et al., 2014). The membrane-anchored isoform can transmit short-range signals through cell-to-cell contacts, whereas soluble SCF can transfer long-range signals through diffusion across the extracellular media (Roskoski, 2005).

The full physiologic functions of the two SCF isoforms is not well understood, however, there is evidence for differential functions in situations such as hematopoiesis, and it appears that both isoforms are necessary for proper mast cell differentiation and survival (Kapur et al., 1998; Lennartsson and Ronnstrand, 2012). In addition to playing a key role in mast cell development, SCF also promotes the proliferation, migration and differentiation of hematopoietic progenitor cells, melanocytes, and germ cells (Lennartsson and Ronnstrand, 2012). A SCF/KIT autocrine loop has been implicated in several human cancers (Ronnstrand, 2004) but is not well studied in non-neoplastic mast cells or canine mast cell tumours.
Tissue-specific homing of murine MCps after release from the bone marrow is dependent upon a number of factors, including mast cell integrin and chemokine receptor expression, specific chemokines, endothelial ligands, and growth factors (Hallgren and Gurish, 2011). For example, the binding of α4β7 integrin to mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) and vascular cell adhesion molecule-1 (VCAM-1) was shown to be critical in the homing of MCps to the intestine (Gurish et al., 2001). One study has also suggested a role for the MCp expression of the thrombopoietin receptor Mpl, in mast cell recruitment to multiple sites, including the dermis, peritoneal cavity and stomach (Ghinassi et al., 2009). The critical mast cell growth factor, SCF, is also a potent KIT-dependent mast cell chemotactic factor whose activity is potentiated by interleukin-3 (IL-3) (Meininger et al., 1992). Homing of MCps from white adipose tissue to intestine and skin also occurs, although the mechanism is not well understood (Poglio et al., 2010).

It has been appreciated for some time that mast cells exist as distinct subpopulations within the body. Careful studies in the 1960s in rats revealed a connective tissue submucosal population of mast cells (connective tissue mast cells, or CTMCs) in sites such as skin and peritoneal cavity that showed differential staining properties compared to atypical mast cells residing in the intestinal lamina propria and between epithelial cells (mucosal mast cells, or MMCs) (Enerback, 1966a, b). Further studies found that the dye-binding differences were related to differential proteoglycan characteristics (Enerback et al., 1985). In contrast to rodents, human mast cells are divided into two major subtypes based on their protease composition: tryptase expression alone (MC\textsubscript{T}), which are located predominantly in mucosa and airway, and tryptase together with chymase expression (MC\textsubscript{TC}), located in skin and perivascular sites (Irani et al., 1986; Schwartz, 2006). In contrast, canine mast cells are classified into three groups: tryptase (MC\textsubscript{T}), chymase (MC\textsubscript{C}) and dual positive (MC\textsubscript{TC}) cells (Noviana et al., 2004). It is now believed that this distinction is an oversimplification (albeit a useful one in understanding mast cell function), and
that mediator content and cell surface receptors of differentiated mast cells can be modulated by interactions with the microenvironment (Galli et al., 2011; Hallgren and Gurish, 2007).

The differentiation of mast cells in their target tissue is dependent upon numerous environmental factors, including cytokines, growth factors, and interactions with the extracellular matrix (Liu et al., 2010; Metcalfe et al., 1997). Once again, SCF plays a critical role in mast cell differentiation and maturation. Bone marrow progenitor cells from humans (Valent et al., 1992), mice (Jamur et al., 2005), and dogs (Lin et al., 2006) cultured with SCF develop into cells that phenotypically and functionally resemble mature mast cells. Numerous interleukins have been shown to induce cellular proliferation and upregulate granulation and mucosal protease levels in mouse and human mast cells; while interferon-γ, transforming growth factor-β, and granulocyte macrophage colony-stimulating factor inhibit cellular proliferation (Okayama and Kawakami, 2006).

**Mast cell structure and function**

After travelling into tissue, mast cells reside there for weeks to months. Mast cell activation can occur in an immunoglobulin E (IgE)-dependent or IgE-independent manner. In the IgE-dependent pathway, which typically occurs in a hypersensitivity reaction, multivalent antigen binds to IgE, which then binds to the Fc portion of FcεRI, on the surface of the mast cell. This leads to receptor aggregation, internalization, and initiates intracellular signalling cascades (Turner and Kinet, 1999). Several different compounds have been shown to activate mast cells in *in vitro* experiments (performed in the absence of IgE) (Galli and Tsai, 2012). Such stimuli include peptides and proteins (e.g. complement cascade products, neuropeptides, chemokines, lipoproteins), chemicals (e.g. opioids), bacteria, lipopolysaccharides (LPS), parasites, toxins, changes in osmolality, and physical stimuli (Table 1.2) (Krishnaswamy et al., 2005; Metz et al., 2007; Theoharides et al., 2012).
Table 1.2 Mast cell stimuli. Mast cells can be activated by numerous physiological and environmental triggers, which can lead to degranulation, or selective release of mediators, without degranulation. Modified from (Theoharides et al., 2012) and (Metz et al., 2007).

<table>
<thead>
<tr>
<th>Stimuli resulting in degranulation</th>
<th>Stimuli resulting in selective release of mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE/Antigen</td>
<td>Viruses (via TLR-3,-5,-9)</td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>LPS (via TLR-4)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Parasites (e.g. <em>Schistosoma mansoni</em>, <em>Leishmania major</em>)</td>
</tr>
<tr>
<td>Hemokinin</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide</td>
<td>Pituitary adenylate cyclase activating polypeptide</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Polychlorinated biphenols</td>
</tr>
<tr>
<td>Endothelin</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>Toxins (e.g. <em>Clostridium difficile</em> Toxin A, Cholera toxin)</td>
</tr>
<tr>
<td>Neuropeptides (e.g. nerve growth factor, substance P)</td>
<td></td>
</tr>
<tr>
<td>Endorphin</td>
<td></td>
</tr>
<tr>
<td>Venoms or venom components</td>
<td></td>
</tr>
<tr>
<td>Physical stimuli (e.g. UV light, cold, heat, pressure, vibration)</td>
<td></td>
</tr>
</tbody>
</table>

IgE, immunoglobulin E; LPS, lipopolysaccharide; TLR, toll-like receptor; UV, ultraviolet

Upon activation, mast cells are stimulated to release many different mediators. Some mediators are preformed and contained within the secretory granules that were first observed over one hundred years ago, while other mediators are newly synthesized. For example, histamine, proteases, proteoglycans, and serotonin are preformed, stored in granules, and can be released through degranulation within minutes upon activation; while lipid-derived mediators, inflammatory cytokines, chemokines, and oxygen-derived free radicals are newly generated and released without degranulation (Figure 1.1). Interestingly, selective release of granule mediators without complete degranulation can also occur (Abdel-Majid and Marshall, 2004; Nakayama et al., 2006; Theoharides et al., 1982). Broadly, the mediators induce acute vascular injury and inflammatory cellular recruitment and adhesion, and in cases of more severe tissue injury, eventual progression to fibrosis, tissue remodeling, and angiogenesis (reviewed in (Beaven, 2009; da Silva et al., 2014; Krishnaswamy et al., 2005)).
Figure 1.1 Mast cell mediated effects. After activation of mast cells, preformed and newly synthesized mediators are released, leading to inflammatory effects, including innate immune responses, as well as adaptive immune responses. Modified from (Krishnaswamy et al., 2005) and (Beaven, 2009).

Mast cells show a diverse range of functions: they play a role in immediate-type (type I) hypersensitivity reactions, both innate and adaptive immunity, tissue homeostasis, chronic inflammatory conditions, and neoplasia. In type I hypersensitivity reactions, upon allergen-induced IgE activation, rapid degranulation of preformed mediators accounts for much of the ‘classic’ acute clinical signs such as increased vascular permeability resulting in tissue edema, in addition to bronchoconstriction and mucous overproduction (da Silva et al., 2014; Gregory and Brown, 2006). The late-phase cutaneous inflammatory response (that peaks by 6 to 12 hours) is also largely attributed to mast cell function, with continued secretion of chemoattractive and
immunomodulatory molecules leading to inflammatory cell recruitment (Barata et al., 1998; Galli et al., 1992).

Much of the early mast cell research focused on its role in allergy; however, new research is revealing how mast cells function in innate and adaptive immunity. Mast cells are able to recognize antigens, toxins, and pathogens through different mechanisms, including the recognition of pathogen-associated molecular patterns (PAMPs) on the surface of the pathogen, the recognition of damage-associated molecular patterns (DAMPs) released by damaged cells, and antibody binding (Brown and Hatfield, 2012; Hofmann and Abraham, 2009). The release of inflammatory mediators results in recruitment of innate immune cells such as eosinophils, natural killer cells, and neutrophils (Burke et al., 2008; Huang et al., 1998; Marshall, 2004). Impressively, mast cells also play a direct role in bacterial clearance, through bacterial phagocytosis (Malaviya et al., 1994), as well as release of antibacterial peptides (Di Nardo et al., 2003). The induction of the adaptive immune responses relies on presentation of antigen to T lymphocytes. Mast cells are not only able to modulate the activation and migration of antigen-presenting cells (APCs) (Reuter et al., 2010), but are also able to process and present antigens themselves in an in vitro setting (Malaviya et al., 1996; Stelekati et al., 2009). Additionally, mast cells can directly enhance T cell activation through the secretion of TNF-α (Nakae et al., 2006; Nakae et al., 2005).

Mast cells also make important contributions towards maintaining tissue function and homeostasis (Maurer et al., 2003). In humans, rodents, and dogs, mast cells are involved in wound healing: in leukocyte recruitment, angiogenesis, granulation tissue formation, and epithelialization (Katayama et al., 1992; Levi-Schaffer and Kupietzky, 1990; Meining and Zetter, 1992; Ng, 2010; Noli and Miolo, 2001; Rock et al., 1990; Weller et al., 2006). Mast cells are important in several aspects of homeostasis such as regulation of hair follicle growth cycles (Maurer et al.,
bone metabolism (Bulfone-Paus and Paus, 2008), and gastrointestinal motility (Van Nassauw et al., 2007).

The multitude of mast cell-mediated effects that make them uniquely poised to combat foreign invaders can also result in serious consequences in a disease setting. In addition to their role in allergy and anaphylaxis (described above), mast cells have also been implicated in the pathogenesis of several human diseases such as Type I diabetes (Geoffrey et al., 2006), interstitial cystitis (Letourneau et al., 1996), bullous pemphigoid (Wintroub et al., 1978), rheumatoid arthritis (Lee et al., 2002), psoriasis (Harvima et al., 1993), atherosclerosis (Bot et al., 2015), and multiple sclerosis (Dines and Powell, 1997; Ibrahim et al., 1996). Mast cells also contribute to persistent inflammation and chronic fibrosis in Crohn’s disease, an inflammatory intestinal disease (Dvorak et al., 1980; Gelbmann et al., 1999), as well as other inflammatory conditions such as asthma, allergic rhinitis, and atopic dermatitis (Brown et al., 2008; Grimbaldeston et al., 2006; Williams and Galli, 2000).

Although much of the work elucidating mast cell development, structure, and function has focused on rodent and human mast cells, canine mast cells have also been studied extensively. Indeed, the cloning of mast cell tryptase and chymase was first reported in the dog (Caughey et al., 1990; Vanderslice et al., 1989). Canine mast cell lines, both with and without c-KIT mutations, have also been utilized to study canine mast cell biology, mast cell neoplasms, as well as the biologic activity of anti-cancer drugs (Gleixner et al., 2007; Lin and London, 2006; Lin et al., 2006; Lin et al., 2009; London et al., 1999; Ma et al., 1999; Thompson et al., 2015). Based on the similarities in biochemical properties of proteases and in characterization of histamine release between canine and human mast cells, canine mast cells have been proposed to be a better model to study the role of mast cells in human disease than rodent mast cells (de Mora et al., 2006). Experimental canine models have been useful in elucidating the role of mast cells in reperfusion in
the lung (Su et al., 1993), small intestine (Szabo et al., 1997), and heart (Frangogiannis et al., 1998; Somasundaram et al., 2005), in intrathecal morphine-induced granulomas (Yaksh et al., 2013), and in mitral valve regurgitation (Stewart et al., 2003). As in humans, mast cells also play a role in the pathogenesis of several diseases in canines, such as atopic dermatitis (Schumann et al., 2014), inflammatory bowel disease (Locher et al., 2001; Woldemeskel et al., 2013), and parvovirus-2-associated enteritis (Woldemeskel et al., 2013).

The relationship between tumours and mast cells is complex. On the one hand, mast cells are able to promote cancer through the release of pro-angiogenic and mitogenic factors and through the degradation of the extracellular matrix (Ch'ng et al., 2006). On the other hand, mast cells can also exert anti-neoplastic effects through cell growth inhibition, promotion of apoptosis, and an anti-tumour inflammatory reaction (Dyduch et al., 2012). These contradictory effects likely explain, at least in part, why mast cell accumulation is associated with a poor prognosis in some human cancers such as melanoma (Reed et al., 1996) and Hodgkin’s lymphoma (Molin et al., 2002), while in some breast cancers, the opposite is true (Dabiri et al., 2004). In addition, dysregulated replication of mast cells themselves can result in neoplasia.

**Mast cell neoplasia**

Mast cell neoplasia results from unregulated clonal proliferation of mast cells. In humans, there are two main variants of proliferative mast cell disease, or mastocytosis, that are usually associated with activating c-**KIT** mutations: 1) cutaneous, which is a skin-limited disease that most commonly affects children; and 2) systemic, with mast cell accumulation in at least one extracutaneous organ (with or without skin involvement) (da Silva et al., 2014; Metcalfe, 2008).

Cutaneous mastocytosis presents as either urticaria pigmentosa, diffuse cutaneous mastocytosis or mastocytomas (da Silva et al., 2014). These diseases show a wide range of symptoms, prognoses are widely variable, and there is no specific cure available (Deho and Monticelli, 2010). In cats,
mast cell tumours (MCTs) occur in three distinct syndromes; cutaneous MCT, splenic/visceral mast cell neoplasia, and intestinal MCT (London and Thamm, 2013). MCTs are the second most common cutaneous tumour in cats and generally occur in older cats (Buerger and Scott, 1987; Miller et al., 1991). Another tumour in young cats of unconfirmed mast cell origin has a histiocytic appearance and will spontaneously regress over time (Chastain et al., 1988; Wilcock et al., 1986). Mast cell neoplasia has also been reported in numerous other domestic species, including ferrets (Poonacha and Hutto, 1984), horses (McEntee, 1991; Millward et al., 2010; Ressel et al., 2015), cattle (Hill et al., 1991), and pigs (Migaki and Langheinrich, 1970). However, the species that is arguably the most severely negatively impacted by MCTs is the dog.

**Canine mast cell tumours**

The MCT is the most common skin tumour of the dog, representing up to 21% of all cutaneous tumours (Bostock, 1986; Cohen et al., 1974; London and Seguin, 2003; Welle et al., 2008). The vast majority of canine MCTs are located in the dermis or subcutaneous tissue; most present as a solitary mass, although 11% to 14% of dogs have multiple synchronous lesions (Bostock, 1973; Hottendorf and Nielsen, 1967; Rothwell et al., 1987). It is imperative to note, that it was not until 2011 that dermal and subcutaneous MCTs were definitively shown to exhibit distinctly different biological behaviour (Thompson et al., 2011a; Thompson et al., 2011b). Therefore, canine MCT studies published prior to 2011 must be interpreted with caution, bearing in mind that if not otherwise stated, they likely included both dermal and subcutaneous MCTs, occurring at a dermal : subcutaneous ratio of approximately 6 : 1 (Sabattini et al., 2015).

Approximately 50% of all solitary cutaneous MCTs occur on the trunk, with the rest occurring on the limbs (40%) and on the head and neck (10%) (Cohen et al., 1974; Hottendorf and Nielsen, 1967). Cutaneous mastocytosis has also rarely been reported in dogs, and the small papules to erythematous plaques that develop resemble urticarial pigmentosa in humans (Davis et
al., 1992; Kato et al., 2016; Pariser and Gram, 2015). There are also occasional reports of primary extracutaneous sites such as conjunctiva, salivary gland, nasopharynx, oral cavity, gastrointestinal tract, ureter, and spine (Iwata et al., 2000; Patnaik et al., 1982; Welle et al., 2008). Visceral forms of MCTs involving the spleen, liver, or bone marrow (known as disseminated or systemic mastocytosis), are uncommon in dogs and have a grave prognosis (Moirano et al., 2017). The visceral form can occur independently of a cutaneous MCT, or, more commonly, is actually a result of widespread metastases of an aggressive cutaneous tumour (O'Keefe et al., 1987; Takahashi et al., 2000). Interestingly, it has been suggested that dogs (with a confirmed cutaneous MCT) with distant metastatic disease, without lymph node metastasis, may have a surprisingly long survival time (Pizzoni et al., 2018).

The clinical appearance of solitary cutaneous MCTs can vary widely, ranging from small, slow growing, well-circumscribed tumours with loss of overlying hair to rapidly growing, ulcerated tumours with surrounding inflammation and small satellite nodules (Bostock, 1986; London and Seguin, 2003; Welle et al., 2008). Importantly, subcutaneous MCTs may be soft and fleshy and can be grossly misdiagnosed as lipomas, a common benign tumour of adipose tissue (Welle et al., 2008). Although MCTs can affect dogs as young as 4 months, they are usually found in older dogs (mean age at presentation of 7.5-9 years) (Thompson et al., 2011a; Welle et al., 2008). There are also certain breed dispositions, with Boxers and Boston Terriers showing an increased incidence of MCTs, although these MCTs are more often lower grade (Bostock, 1986; Brodey, 1970; Villamil et al., 2011). Similarly, Boxers tend to have less aggressive subcutaneous MCTs compared to other breeds (Thompson et al., 2011a). Despite the paucity of large-scale breed-specific studies, the numerous smaller studies showing associations between breed and incidence, site predilections, and tumour behaviour suggest that MCT development has a genetic component (Peters, 1969; Welle et al., 2008). Other factors that have been hypothesized to play a
role in MCT etiology include chronic inflammation, skin irritants, and viruses; however, conclusive studies to support these hypotheses are still lacking (London and Thamm, 2013).

The myriad of functional effects of mast cells lends itself to numerous complications arising secondary to MCTs. Occasionally, physical examination of the tumour induces mast cell degranulation, resulting in erythema and wheal formation (known as Darrier’s sign) (London and Seguin, 2003). Histamine release from MCTs can lead to gastrointestinal ulceration with 35% to 83% of dogs with MCTs showing evidence of gastrointestinal ulceration at necropsy (Howard et al., 1969), and rarely, systemic anaphylaxis and collapse (Blackwood et al., 2012). Other potential complications include delayed wound healing after surgical excision, coagulation defects in blood around MCTs, in addition to lesions often related to gastrointestinal ulceration such as anorexia, vomiting, melena, anemia, and rarely, intestinal perforation and peritonitis (Welle et al., 2008).

Diagnosis of solitary MCTs can be made with cytological or histopathological examination. Cytological examination of fine-needle aspirations stained with a Wright-Giemsa stain, revealing a round-cell population with purplish red (metachromatic) cytoplasmic granules, yielded a correct diagnosis in approximately 95% of confirmed MCTs (including both well-granulated and poorly granulating tumours) (Baker-Gabb et al., 2003). Some general histological features of canine cutaneous MCTs include an unencapsulated nodule in the dermis and/or subcutaneous tissue, composed of discrete round cells arranged in rows or loose sheets (Patnaik et al., 1984; Thompson et al., 2011a). The cells have a central round nucleus and variable numbers of basophilic cytoplasmic granules that stain metachromatically with metachromatic stains such as toluidine blue or Giemsa. There are variable numbers of eosinophils scattered throughout the MCT or forming aggregations (Welle et al., 2008). The surrounding collagen may be fibrotic, edematous or hyalinized, and may show collagen degeneration; tissue necrosis may also be present (Patnaik et al., 1984; Welle et al., 2008). The wide variety of functional properties of mast cells and their
dynamic cross talk with the microenvironment may account for the variability seen in the stroma surrounding MCTs.

**Prognosis of canine mast cell tumours**

The ability to accurately predict a tumour’s behaviour is critical in directing patient therapy, and is especially important in canine MCTs as their behaviour is highly variable, ranging from benign to a fatal systemic disease (O'Connell and Thomson, 2013). Factors shown to have statistically significant correlations with clinical outcome (albeit with variable clinically useful significance) include cell proliferation rate, biomarker expression pattern, histologic architectural pattern, location of tumour, clinical appearance, growth rate, size, the presence of systemic signs, breed, sex and clinical stage (i.e. presence of lymph node involvement or distant metastasis) (Table 1.3) (Berlato et al., 2018; Da Silva et al., 2017; Horta et al., 2018; London and Thamm, 2013; Pulz et al., 2017; Welle et al., 2008). A recent study has shown that cytologic grade may be useful in directing treatment and for prognostication (Camus et al., 2016). However, histologic grade is still generally considered the most consistent and reliable prognostic indicator, although it does not predict the behaviour of every tumour (Bostock, 1973; Kiupel et al., 2011; Murphy et al., 2004; Patnaik et al., 1984). Pre-treatment incisional biopsies are useful in determining grade, as their grade has a high level of correlation with the grade evaluated from excisional biopsies (Shaw et al., 2017).
Table 1.3 *Canine mast cell tumour prognostication factors*. Many factors (with variable predictive ability) have been associated with better prognosis (positive prognostic factor) or poorer prognosis (negative prognostic factor). Modified from (London and Thamm, 2013).

<table>
<thead>
<tr>
<th>Positive prognostic factor</th>
<th>Negative prognostic factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic grade</td>
<td></td>
</tr>
<tr>
<td>Patnaik grade I*</td>
<td>Patnaik grade III*</td>
</tr>
<tr>
<td>Kiupel low-grade**</td>
<td>Kiupel high-grade**</td>
</tr>
<tr>
<td>Cell proliferation rate</td>
<td></td>
</tr>
<tr>
<td>Low mitotic count, low levels of Ki67 and AgNORs</td>
<td>High mitotic count, high levels of Ki67 and AgNORs</td>
</tr>
<tr>
<td>c-KIT mutation status</td>
<td></td>
</tr>
<tr>
<td>Absence of activating mutation</td>
<td>Presence of activating mutation</td>
</tr>
<tr>
<td>KIT localization</td>
<td></td>
</tr>
<tr>
<td>Membrane</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>KIT phosphorylation</td>
<td></td>
</tr>
<tr>
<td>Low phosphorylation</td>
<td>High phosphorylation</td>
</tr>
<tr>
<td>VEGFR2/pVEGFR2 expression by neoplastic cells</td>
<td></td>
</tr>
<tr>
<td>Low expression</td>
<td>High expression</td>
</tr>
<tr>
<td>c-CBL</td>
<td></td>
</tr>
<tr>
<td>Low expression</td>
<td>High expression</td>
</tr>
<tr>
<td>Architectural pattern</td>
<td></td>
</tr>
<tr>
<td>Cords</td>
<td>Sheets</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
</tr>
<tr>
<td>Single site</td>
<td>Lymph node involvement or distant metastasis</td>
</tr>
<tr>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>Mucous membrane; preputial and scrotal tumours; visceral and bone marrow disease</td>
</tr>
<tr>
<td>Growth rate</td>
<td></td>
</tr>
<tr>
<td>Slow growth without significant change (present for months or years) are usually benign</td>
<td>Rapid growth</td>
</tr>
<tr>
<td>DNA ploidy</td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>Aneuploid may be associated with shorter survival times and higher-stage disease</td>
</tr>
<tr>
<td>Microvessel density</td>
<td></td>
</tr>
<tr>
<td>Decreased density</td>
<td>Increased density</td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
</tr>
<tr>
<td>No recurrence following surgical excision</td>
<td>Local recurrence following surgical excision</td>
</tr>
<tr>
<td>Systemic signs</td>
<td></td>
</tr>
<tr>
<td>No systemic signs</td>
<td>Presence of systemic signs (e.g. vomiting, GI ulceration, melena) may be associated with worse prognosis</td>
</tr>
<tr>
<td>Tumour size</td>
<td></td>
</tr>
<tr>
<td>Small tumours (more amenable to complete excision)</td>
<td>Large tumours may be associated with worse prognosis after surgical removal and/or RT</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Younger dogs</td>
<td>Older dogs may be associated with shorter median DFIs after RT</td>
</tr>
<tr>
<td>Breed</td>
<td></td>
</tr>
<tr>
<td>Brachycephalic breeds tend to have low-grade tumours</td>
<td>Non-brachycephalic breeds</td>
</tr>
</tbody>
</table>

AgNORs, argyrophilic nucleolar organizer regions; DFI, disease-free interval; GI, gastrointestinal; RT, radiation therapy; VEGFR2, vascular endothelial growth factor receptor 2
* (Patnaik et al., 1984)
** (Kiupel et al., 2011)
A widely adopted canine cutaneous MCT histopathologic grading scheme was published by Patnaik and colleagues in 1984 (Northrup et al., 2005b; Patnaik et al., 1984; Welle et al., 2008). This scheme compared histomorphologic features to define three grades of tumour. Grade I tumours included well-differentiated tumours that were confined to the dermis with no observed mitotic figures in the section examined, grade II tumours included intermediately differentiated tumours infiltrating or replacing the lower dermal and subcutaneous tissue, with 0 to 2 mitotic figures per 400X high power field (HPF) (although there was no indication of how many HPFs are needed to confirm this number), and grade III tumours included highly cellular, poorly differentiated tumours with replacement of subcutaneous and deep tissues, and 3 to 6 mitotic figures per HPF. The study was able to separate clinically benign from highly aggressive tumours, as evidenced by the fact that grade I tumours showed much higher survival rates (93% alive at 1500 days) compared to grade III tumours (6% alive at 1500 days). Furthermore, grade III tumours were reported to have the highest metastatic rate, at 55% to 96% of cases (Thamm et al., 2003).

Notably, the Patnaik et al. paper was one of the few early studies to separate dermal from subcutaneous MCTs. In this study, they excluded subcutaneous MCTs, providing a scheme that could be used solely for dermal MCTs. Unfortunately, veterinary pathologists unaware of this caveat would automatically classify subcutaneous MCTs as grade II or III, possibly resulting in more aggressive treatment than necessary. In recent years, subcutaneous MCTs have been studied and characterized (see subsection: Cutaneous versus subcutaneous MCTs).

Despite the significant advance the Patnaik scheme represented, there were two major limitations in the study. First, there is large interobserver variation, which may be partially related to the lack of precisely defined criteria in which to assign grades. In one instance, ten veterinary pathologists grading 60 cutaneous MCTs showed a mean agreement of only 62%, prompting them to conclude that a more objective grading scheme is required (Northrup et al., 2005b).
Concordance in another study was 75% for grade III MCTs, but less than 64% for grades I and II (Kiupel et al., 2011). Second, the Patnaik scheme is quite poor in predicting outcome for grade II tumours, which can represent a large proportion (60 - 75%) of dermal MCTs (Sabattini et al., 2015; Stefanello et al., 2015). Although many dermal grade II MCTs behave in a benign fashion, 17% of cases showed metastatic disease (Stefanello et al., 2015), while the mortality rate due to MCT-associated disease was 12% (Sabattini et al., 2015). This may be related to the grade II classification of potentially highly aggressive tumours that do not meet the requirement of the rather high mitotic count of 3 mitotic figures per HPF (Kiupel et al., 2011).

Several other schemes have been used by veterinary pathologists to classify MCTs, some of which allow for subcutaneous tumours to be classified as low-grade (Northrup et al., 2005a; Pulley and Stannard, 1990; Walder and Gross, 1992; Yager and Wilcock, 1994). However, one cutaneous MCT grading scheme that has become more commonly used in recent years was published in 2011 by Kiupel and colleagues (Kiupel et al., 2011). This 2-tier system was specifically designed to improve concordance and prognostication of the Patnaik scheme. A high-grade MCT is defined as having at least one of the following: 1) at least 7 mitotic figures in 10 400X HPFs; 2) at least 3 multinucleated cells in ten 400 X HPFs; 3) at least 3 bizarre nuclei in 10 400X HPFs; or 4) karyomegaly. Notably, there are no criteria for tumour location and/or tissue invasion.

Put to the test, Kiupel and colleagues reported 97% consistency with their grading scheme, with a median survival time of less than 4 months for high-grade MCTs, and greater than 2 years for low-grade MCTs (which represented approximately 90% of the studied tumours) (Kiupel et al., 2011). Further studies confirmed improved consensus grading (Takeuchi et al., 2013; Vascellari et al., 2013), with two reports classifying all Patnaik grade I tumours as Kiupel low-grade, all grade III tumours as high-grade, and approximately 85% of grade II tumours as low-grade (Sabattini et
al., 2015; Stefanello et al., 2015). Although this grading scheme shows improvement over the Patnaik grading scheme, the one unfortunate drawback is that both dermal and subcutaneous MCTs were included. Exactly how this lack of separation has influenced the prognostic ability of the Kiupel scheme is difficult to determine. Importantly, evaluation of molecular markers in addition to the grading schemes was still proposed by Kiupel and others for improved prognostication (Kiupel et al., 2011; Sabattini et al., 2015).

Simple evaluation of mitotic count, even independent of a grading scheme, is a strong prognostic factor (Elston et al., 2009; Romansik et al., 2007; Thompson et al., 2011a; Thompson et al., 2011b). A more sensitive measure of proliferation is measurement of the cell proliferation marker, Ki67, a nuclear protein whose levels correlate with cell proliferation (Gerdes et al., 1984). The Ki67 score was reported to be significantly higher in dogs that died of cutaneous MCTs (Abadie et al., 1999; Webster et al., 2007), and has been useful in subdividing Patnaik grade II tumours into two groups associated with different survival times (Abadie et al., 1999; Scase et al., 2006). The frequency of another proliferation marker, agyrophilic nucleolar organizer regions (AgNORs), has also been shown to be predictive of aggressive biologic behaviour in cutaneous MCTs (Bostock et al., 1989), while a third, proliferating cell nuclear antigen (PCNA) appears to be less reliable (Bostock et al., 1989; Simoes et al., 1994).

Several additional potential prognostic markers have been investigated, including ploidy status (Ayl et al., 1992), expression of the tumour-suppressor protein p53 (Ginn et al., 2000; Jaffe et al., 2000), nuclear morphometric analysis (Maiolino et al., 2005; Strefezzi Rde et al., 2003), intratumoural microvessel density (Preziosi et al., 2004), expression of p27 and p21 (Wu et al., 2004), matrix metalloproteinases (Giantin et al., 2012a), cyclooxygenase-2 (Prada et al., 2012; Vascellari et al., 2013), interleukin-2 and its receptor (Meyer et al., 2012, 2013), bcl-2 and its protein family members (Strefezzi Rde et al., 2012; Vascellari et al., 2013), p62/sequestosome-1
(Rich et al., 2015), and micro-RNA-9 (Fenger et al., 2014). A recent transcriptome analysis of MCTs also identified a set of 13 differentially expressed transcripts between differentiated and undifferentiated MCTs (Giantin et al., 2014). Of all the studied markers, it has been suggested that KIT staining is currently the most useful prognostic and therapeutic marker (Gil da Costa, 2015).

Immunohistochemical staining of KIT has been evaluated in MCT cells and distinct localization patterns have been identified; membranous, focal/stippled cytoplasmic, and diffuse cytoplasmic (Kiupel et al., 2004). While non-neoplastic mast cells show membranous KIT localization, the presence of neoplastic mast cells with a diffuse cytoplasmic pattern correlated with increased rates of local recurrence and decreased survival (Kiupel et al., 2004; Thompson et al., 2015; Webster et al., 2007). We have also recently reported that both phosphorylated KIT and expression of total and phosphorylated VEGFR2, both receptor tyrosine kinases (RTKs), are predictive of poorer clinical outcomes (Da Silva et al., 2017; Thompson et al., 2015). In addition, neoplastic mast cell expression of c-casitas B-lineage lymphoma (c-CBL), an E3 ubiquitin ligase, was associated with reduced disease-free survival (Da Silva et al., 2017). Somatic activating mutations within the juxtamembrane domain of the c-Kit gene have also been associated with more aggressive disease in cutaneous MCTs (Downing et al., 2002; Zemke et al., 2002), although c-Kit mutations are reported in less than 35% of cases in studies that included sequencing of exons 8 to 13 and 17 to 19 (Letard et al., 2008) or the entire open reading frame (Takeuchi et al., 2013). There have to date been no internal tandem duplication mutations reported in exon 11 in subcutaneous MCTs (Thompson et al., 2011b).

**Dermal versus subcutaneous mast cell tumours**

The Patnaik grading scheme attributed great importance to tumour location/infiltration in assigning grade and predicting tumour behaviour (Patnaik et al., 1984). However, a perplexing report some years later appeared to show no association between tumour depth and survival rate
These inconsistent results may be attributed to the failure of the latter study to recognize dermal and subcutaneous MCTs as separate entities, and erroneously applying the Patnaik scheme to subcutaneous tumours. It was not until 2007 that a preliminary report suggested subcutaneous tumours were distinct MCT variants, and were associated with longer survival times (Newman et al., 2007).

Our laboratory conducted a statistically powerful study of over 300 subcutaneous MCTs with known clinical outcomes that revealed that the majority of subcutaneous tumours had a favourable outcome (Thompson et al., 2011a). Additionally, mitotic count was shown to be a strong predictor of local recurrence, metastasis and survival rates (Thompson et al., 2011a). Further investigations using case-control designs to match benign and aggressive subcutaneous tumours showed that higher counts of Ki67 and AgNOR, and a diffuse cytoplasmic KIT localization pattern were also associated with poorer outcomes (Thompson et al., 2011b). A recent report has confirmed that subcutaneous MCTs are largely benign, with a mortality rate due to MCT-associated disease of only 8% (Sabattini et al., 2015). Considering that the microenvironment plays an important role in normal mast cell development, it is possible that subcutaneous neoplastic mast cells possess phenotypic and functional differences compared to neoplastic mast cells developing within the dermis, or that surrounding tissue (e.g. adipose tissue) is influencing tumour progression resulting in less aggressive behaviour. Another intriguing hypothesis is that the mast cells forming subcutaneous tumours actually arise from progenitor cells found not in the bone marrow, but in white adipose tissue (Thompson et al., 2011a).

**Treatment of canine mast cell tumours**

Several different factors dictate the recommended treatment for MCT patients. These factors include whether the site is amenable to wide surgical excision, the histologic grade, and evaluation of spread of the disease, which is now most commonly done through fine needle
aspiration of the regional lymph node, and staging by an abdominal ultrasound (London and Thamm, 2013). An important consideration, however, is that dogs often have low numbers of mast cells in lymph nodes, which can increase in number in the presence of dermal ulceration. Indeed, mast cells were present in lymph node aspirates from approximately one quarter of healthy dogs (Bookbinder et al., 1992). Clustering and aggregations of mast cells in lymph node aspirates are more indicative of metastatic spread (Krick et al., 2009). Histopathology of lymph nodes showing aggregates or sheets of mast cells is considered evidence of metastasis, and correlates with shorter survival time (Weishaar et al., 2014).

The optimal treatment algorithm differs between grade I/localized grade II MCTs versus metastasized grade II/grade III MCTs (summarized in (London and Thamm, 2013)). Briefly, some of the main differences include the use of adjunctive medical and/or radiation therapy in completely excised grade III tumours and incompletely excised grade I and II tumours, in contrast to simple routine follow up physical examinations for completely excised localized grade I or II tumours. The use of the term “complete excision” varies depending on the histologic grade; 3 cm margins are typically the target for MCTs, but recent evidence suggests that 1 to 2 cm margins are sufficient for grade I and II tumours (Fulcher et al., 2006; Simpson et al., 2004).

Currently, options for adjunctive medical therapy include oral corticosteroids such as prednisone, as well as cytotoxic chemotherapy drugs, such as vinblastine and lomustine, and radiation to control local recurrence (London and Thamm, 2013). The effects of several new treatments have recently been analyzed in pilot or in vitro studies, including oncolytic sendai virus therapy (Ilyinskaya et al., 2018), targeting the c-KIT promoter through the use of G-quadruplex ligands to downregulate KIT expression (Zorzan et al., 2018), administration of heat shock protein 90 inhibitors to downregulate KIT (London et al., 2018), electrochemotherapy with IL-12 gene (which encodes a pleiotropic cytokine) electrotransfer to induce an inflammatory response against
neoplastic cells (Cemazar et al., 2017; Salvadori et al., 2017), intrallesional injection of the glucocorticoid triamcinolone (Case and Burgess, 2018), and inhibitors of the JAK2/STAT5 pathway to inhibit neoplastic cell proliferation and survival (Keller et al., 2018). It has also recently been shown that removal of regional lymph nodes with confirmed metastases may have therapeutic value and reduce the risk of tumour progression and MCT-related death (Marconato et al., 2018).

As previously discussed, the RTK KIT plays an important role in normal mast cell development and can show dysregulation in MCTs. The use of medications that inhibit signaling through KIT, called small molecule RTK inhibitors, is proving to be extremely powerful in treating canine MCTs. Interestingly, not only has response to treatment been reported in those tumours with c-KIT activating mutations, but also a large number of those that do not (London et al., 2009; Weishaar et al., 2018) (see subsection: Tyrosine kinase inhibitors in canine MCTs). A novel anti-KIT antibody has also shown to be a powerful inhibitor of both wildtype and mutant KIT in vitro, and reduced cutaneous mast cell numbers in dogs with MCTs in a phase I clinical trial (London et al., 2017).

**Receptor tyrosine kinases**

Polypeptide growth factors and cytokines are vital extracellular signals in promoting cell proliferation and differentiation, and cell survival and metabolism. Receptor tyrosine kinases (RTKs) are a family of high-affinity cell surface receptors that mediate many of the effects of these growth factors. Although RTKs are numerous and diverse, they share certain features: all RTKs are transmembrane proteins that bind ligands through their extracellular domains resulting in activation of the tyrosine kinase located in the cytoplasmic domain (Fantl et al., 1993). Tyrosine kinases are enzymes that transfer phosphate groups from adenosine triphosphate (ATP) to a tyrosine residue of a target molecule (Roskoski, 2005). These phosphorylation events lead to
activation of various intracellular signaling cascades, the nature of which depends on the specific RTK as well as the cell type (Fantl et al., 1993). In humans, there are 58 identified RTKs, which based on structural characteristics, are classified into 20 subfamilies (Lemmon and Schlessinger, 2010). Many key features of RTK structure and function are highly conserved from the nematode Caenorhabditis elegans to humans (Lemmon and Schlessinger, 2010).

KIT structure and function

In 1986, a viral oncogene from the Hardy-Zuckerman 4 feline sarcoma virus was identified and named v-KIT (Besmer et al., 1986). Shortly thereafter, c-KIT, the normal mammalian cellular homologue was cloned and sequenced (Yarden et al., 1987). The encoded protein, KIT, belongs to the type III subfamily of RTKs, which also includes two platelet-derived growth factor (PDGF) receptors (α and β), the macrophage colony stimulating-factor receptor (CSF-1R), and the Fl cytokine receptor (Flt3/Flk2) (Lemmon and Schlessinger, 2010). The members of this subfamily are characterized by the presence of five immunoglobulin (Ig)-like extracellular domain repeats, a transmembrane region, an intracellular juxtamembrane region, an intracellular kinase domain that is split by a kinase insert sequence of 70-100 amino acids located near the middle of the domain, and a C-terminal tail (Figure 1.2,A) (Lennartsson and Ronnstrand, 2012).

KIT activation requires dimerization. This can occur rapidly, with dimers forming within minutes after ligand addition (Broudy et al., 1998). Stem cell factor is a homodimer and one molecule of SCF can bind tightly to the first three Ig-like domains in KIT (Yuzawa et al., 2007). KIT dimerization is achieved when two KIT receptors are each bound to one molecule of a SCF homodimer at the same time (Figure 1.2,B) (Lemmon et al., 1997). When the two KIT monomers come together, conformation changes and homotypic interactions occur between the Ig-like domains 4 and 5 (Yuzawa et al., 2007). This brings the transmembrane regions as well as the intracellular kinase domains of the two KIT receptors into close proximity.
Figure 1.2 Schematic representation of KIT. (A) The KIT protein contains 5 Ig-like extracellular domains. Ig-like domains 1-3 bind SCF (unbound SCF homodimer is shown in blue box), and Ig-like domains 4 and 5 are involved in the dimerization process. There is a single transmembrane domain, and split tyrosine kinase domain, in between which is the regulatory juxtamembrane domain. The last 50 amino acids form the carboxyterminal tail. (B) The binding of SCF brings two KIT monomers close together, allowing for interactions between the Ig-like domains 4 and 5 which stabilize formation of KIT homodimers. The juxtamembrane region is phosphorylated through trans-phosphorylation, with subsequent phosphorylation of the kinase insert region, kinase domain, and carboxyterminal tail. Adapted from (Lennartsson and Ronnstrand, 2012).

Ig, immunoglobulin; SCF, stem cell factor

The kinase domain contains an NH2-terminal lobe and a COOH-terminal lobe, between which is located the active site (Mol et al., 2003). In an inactive state, the juxtamembrane domain forms a hairpin loop between the lobes and inhibits enzymatic activity (Mol et al., 2004). After dimerization, two tyrosine residues within the juxtamembrane domain become phosphorylated through trans-phosphorylation, which releases the juxtamembrane domain from the kinase domain and enables catalytic function (Mol et al., 2004; Mol et al., 2003). Full activation of the kinase
domain occurs through autophosphorylation of up to ten more tyrosines within the intracellular domain in an orderly manner (DiNitto et al., 2010; Lennartsson et al., 2005). Most of the phosphorylated tyrosine residues act as docking sites for signaling molecules with Src homology 2 domains, which can initiate several well-established downstream signal transduction pathways, including phosphatidylinositol 3 (PI3)-kinase, Src family kinase, mitogen-activated protein kinase (MAPK) pathways, and phospholipases (Lennartsson and Ronnstrand, 2012).

In order to modulate the duration and intensity of the KIT signaling pathways, mechanisms must be in place to downregulate KIT activation. Attenuation of KIT signaling is possible through ubiquitination, with internalization and degradation of KIT receptors (Masson et al., 2006), inactivation of the kinase domain through serine phosphorylation of the kinase insert region (Blume-Jensen et al., 1995), and tyrosine dephosphorylation (Paulson et al., 1996). In human disease, constitutive KIT activation has been implicated in the pathogenesis of systemic mastocytosis (Bodemer et al., 2010; Hanssens et al., 2014), gastrointestinal stromal tumours (Hirota et al., 1998), leukemia (Corbacioglu et al., 2006; Wang et al., 2005), and prostate cancer (Wiesner et al., 2008).

**KIT expression and dsyregulation in canine mast cell tumours**

The amino acid sequence of canine KIT is highly conserved, showing 93%, 88%, and 81% identity when compared to feline, human, and murine KIT, respectively (Ma et al., 1999). While the extracellular domain is 78% identical between canine and human KIT, the intracellular domain is 97% identical, with perfect conservation of all 38 residues in the regulatory juxtamembrane region (Ma et al., 1999), and the phospho-transferase portion of the kinase domain is 99.5% identical, with conservation of all tyrosine residues (Webster et al., 2006). KIT is expressed by a number of different cell types in the adult dog, including cerebellar Purkinje cells, mammary gland duct and acinar cells, interstitial cells of Cajal in the gastrointestinal tract, endometrial epithelial
cells, and of course, mast cells (Morini et al., 2004). Several canine neoplasms also show expression of KIT, including gastrointestinal stromal tumours (Bettini et al., 2003), as well as mammary gland (Morini et al., 2004), ovarian (Morini et al., 2004), testicular (Morini et al., 2004; Reis-Filho et al., 2004), renal cell (Gil da Costa et al., 2011), and Merkel cell tumours (Gil da Costa et al., 2010), cutaneous melanomas and melanocytomas (Gomes et al., 2012), and of course, MCTs (London et al., 1996; Morini et al., 2004).

Most of the known activating c-KIT mutations in canine dermal MCTs occur within exon 11, which encodes the regulatory juxtamembrane domain, with reported rates of 8 to 17% of MCTs across all histological grades (Giantin et al., 2012b; Takeuchi et al., 2013; Webster et al., 2006; Zemke et al., 2002), and 35% in Patnaik grade II or III tumours (Downing et al., 2002). Exon 11 mutations, which often consist of tandem duplications, inhibit the regulatory function of the juxtamembrane domain, resulting in constitutive, SCF-independent KIT activation (London et al., 1999; Ma et al., 1999). Mutations within other exons have also been reported, including exons 8 and 9, which can also lead to constitutive KIT phosphorylation, however these mutations are much less studied (Letard et al., 2008). The presence of exon 11 c-KIT mutations correlates with higher histological grade (Zemke et al., 2002), increased risk of local and systemic recurrence (Webster et al., 2006), a shorter overall survival and progression-free survival (Takeuchi et al., 2013), and upregulation of cytoskeleton structure or cell motility proteins (Schlieben et al., 2013). To date, investigations of subcutaneous MCTs have only revealed wildtype c-KIT by sequencing exon 11 and the 5′ end of intron 11 (Thompson et al., 2011b).

As previously mentioned, there are three distinct KIT staining patterns observed in canine MCT cells, namely membranous, focal/stippled cytoplasmic, and diffuse cytoplasmic. Normal mast cells show membranous KIT staining, whereas neoplastic cells may show a focal or diffuse cytoplasmic pattern that correlates with activating c-KIT mutations (Webster et al., 2006).
Aberrant KIT protein localization is also associated with increased tumour recurrence and reduced post-surgical survival (Kiupel et al., 2004; Thompson et al., 2015; Webster et al., 2007), higher histological grade and increased cell proliferation markers (Gil da Costa et al., 2007) in dermal MCTs, and increased recurrence and metastases in subcutaneous MCTs (Thompson et al., 2011b). Interestingly, KIT staining levels do not correlate with altered KIT staining patterns (Giantin et al., 2012b; Webster et al., 2006), suggesting that cytoplasmic staining patterns may result from abnormal post-translational modifications rather than increased transcription levels.

**Tyrosine kinase inhibitors in canine mast cell tumours**

There are two orally bioavailable RTK inhibitors that inhibit KIT and are approved for use in veterinary medicine: toceranib (Palladia; Pfizer Animal Health), and masitinib (Kinavet; AB Science) (London and Thamm, 2013). A large multicenter study evaluating toceranib treatment in dogs with recurrent, Patnaik grade II or III MCTs showed a response rate of 69% in MCTs harboring c-KIT mutations (London et al., 2009). Interestingly, in MCTs without mutations, 37% of cases showed a response. Another study evaluating the long-term outcome of dogs with nonresectable grade II or III MCTs treated with masitinib showed increased time to tumour progression compared with placebo regardless of c-KIT mutational status (Hahn et al., 2010). In addition, 10% of dogs with wild-type c-KIT and 33% with mutated c-KIT had a complete tumour response at 12 months. Another recent study looking at MCTs treated with toceranib or vinblastine, found that c-KIT mutational status did not predict treatment response with either treatment (Weishaar et al., 2018).

Exactly why a subset of the wild-type MCTs responded to RTK treatment is not clear. However, it is tempting to hypothesize that it is related to the fact that toceranib and masitinib also inhibit other RTKs, such as VEGFR2 and/or PDFGRα/β (London and Thamm, 2013), which may also be playing important roles in tumour progression, in both neoplastic cells, as well as in
stromal cells. There is evidence of VEGF expression in canine MCTs, with stronger expression in grade III tumours (Giantin et al., 2012a; Patruno et al., 2009). Two preliminary studies found cell surface expression of VEGFR-1 and cytoplasmic expression of VEGFR-2 (Rebuzzi et al., 2007; Sekis et al., 2009). Recently, expression of VEGFR2 in a subset of canine dermal and subcutaneous MCTs has been demonstrated by Western blot analysis (Thompson et al., 2015). Moreover, VEGFR2 expression was a risk factor for decreased survival, increased metastasis and local recurrence. An analysis of PDGFRα/β also showed expression of PDGFRβ in a subset of tumours, but this was not determined to be a significant risk factor. In another study examining 14 subcutaneous MCTs, cases with high expression of VEGFR2 and phosphorylated VEGFR2 in neoplastic cells were significantly associated with reduced disease free survival (Da Silva et al., 2017).

One of the major limitations of RTK inhibitor therapy is the development of resistance that often arises during long-term treatment (London and Thamm, 2013). The mechanism of resistance in canine MCTs is not known. A recent study has provided some insight, reporting that toceranib-resistant c-KIT mutant MCT cell lines developed secondary c-KIT mutations in the juxtamembrane and tyrosine kinase domains and showed overexpression of c-KIT mRNA and KIT protein (Halsey et al., 2014). A transcriptome analysis in a masitinib-treated MCT-derived cell line showed upregulation of several alternative pro-proliferative pathways. This may help explain the development of resistance (Klopfleisch et al., 2012).

**Protein degradation pathways**

The proper maintenance of healthy proteins in cells is important for cell metabolism, organelle biogenesis, and adaptation to external and internal stressors. An important part of this maintenance involves the degradation of misfolded or damaged proteins that can impair cellular
functions. Two major degradation pathways exist in cells, the autophagy-lysosome pathway, and the ubiquitin-proteasome system.

**Autophagy**

Autophagy occurs continuously in healthy cells to eliminate and recycle damaged intracellular constituents. The first step in autophagy is nucleation, whereby multiple proteins assemble to form a phagophore. In the next step, the phagophore elongates, curves around, and seals itself to form the autophagosome. The last step involves fusion of the autophagosome with the lysosome and subsequent degradation. Autophagy is able to degrade not only proteins, but also organelles (Hurley and Young, 2017). By recycling organelles during times of starvation, autophagy is able to replenish the cells with biosynthetic precursors and energy sources. This may also lead to autophagy-associated cell death, which is important for development, as well as tissue survival and cellular remodeling in times of environmental stress.

There is increasing evidence that autophagy is dysregulated in neoplasia (Ozpolat and Benbrook, 2015). The protein beclin-1 plays a key role in the nucleation step of autophagy and is encoded by a putative tumour suppressor gene, BECN1 (Liang et al., 1999). Monoallelic deletion of the BECN1 gene is found in several human cancers and is thought to contribute to tumourigenesis (Gao et al., 1995; Russell et al., 1990; Saito et al., 1993). Knockdown of BECN1 leads to accumulation of KIT protein in human gastrointestinal stromal tumour cell lines driven by mutant c-KIT (Hsueh et al., 2013). Conversely, cancer cells can also exploit the process of autophagy in order to survive in the hypoxic, acidic, and poor nutrient tumour microenvironment (Ozpolat and Benbrook, 2015). Importantly, not only is autophagy induced in the metabolically challenged tumour microenvironment, but also in response to radiation therapy and anticancer treatments, including RTK inhibitors (Abedin et al., 2007; Amaravadi et al., 2007; Han et al., 2011; Paglin et al., 2001; Rouschop and Wouters, 2009). Several clinical trials are currently
underway to investigate the effect of autophagy inhibitors in combination with chemotherapies in various human cancers (Ozpolat and Benbrook, 2015).

The role of beclin-1 in the pathogenesis of canine MCTs is not well understood. In malignant canine mammary tumours, cytoplasmic beclin-1 expression was reported to be lower than in surrounding normal mammary glands, and loss of beclin-1 expression was associated with poor overall survival (Liu et al., 2013). One hint that autophagy may be important in MCT biology comes from a recent study examining p62/sequestosome-1, a “hub” protein that plays a role in apoptosis and protein delivery to autophagosomes (Rich et al., 2015). The study showed p62 nuclear immunoreactivity was significantly associated with Kiupel low-grade tumours, and p62 cytoplasmic immunoreactivity was significantly associated with high-grade tumours (Rich et al., 2015).

**Ubiquitin proteasome system**

The ubiquitin proteasome system is the primary protein degradation pathway for short-lived proteins that are unfolded, misfolded or damaged, or that otherwise require rapid regulated destruction (Dikic, 2017). Protein ubiquitination involves three types of enzymes: E1, the ubiquitin-activating enzyme; E2, a ubiquitin-conjugating enzyme; and E3, a ubiquitin ligase. Polyubiquitinated proteins are targeted for destruction through delivery to and degradation within the proteasome (Cohen-Kaplan et al., 2016).

The casitas B-lineage lymphoma (CBL) proteins (c-CBL, CBL-B, and CBL-3) are a highly conserved family of E3 ubiquitin protein ligases that ubiquitinate and target many RTKs, including KIT, VEGFR2 and PDGFRs, for degradation within the proteasome (Masson et al., 2006; Ryan et al., 2006; Zeng et al., 2005). Mutations in CBL genes result in the deregulation of RTKs (Peschard and Park, 2003). Human acute myeloid leukemia cell lines with CBL mutations show strong surface expression of KIT, presumably due to decreased receptor ubiquitination (Makishima et al.,
In a murine myeloid cell line, the presence of mutant CBL conferred SCF-independent growth (Bandi et al., 2009). Furthermore, these mutant CBL genes induced a generalized mastocytosis and myeloproliferative disease after retroviral transduction into murine bone marrow cells in vivo (Bandi et al., 2009). Interestingly, the transformation induced by mutant CBL appears to be dependent on the presence of KIT and on Src family kinases (SFKs), and independent of KIT kinase activity (Bandi et al., 2009). This may explain the much larger growth inhibitory effect of dasatinib (dual SFK and RTK inhibitor) compared to imatinib (KIT inhibitor) (Bandi et al., 2009; Makishima et al., 2012).

In a study of canine subcutaneous MCTs, high levels of neoplastic cell c-CBL immunoreactivity were significantly associated with reduced disease-free survival (Da Silva et al., 2017). Given that combination therapy of a proteasome inhibitor (that induced degradation of KIT by binding KIT to CBL) and dasatinib stimulated KIT internalization-induced apoptosis of human gastrointestinal stromal cells (Dong et al., 2015), investigations into the role of protein degradation pathways in canine MCTs hold much promise.

**Tissue microarrays**

A tissue microarray (TMA) is an array-based technique that enables high-throughput staining profiling of large numbers of tissues or tumours (Kononen et al., 1998). A typical TMA is composed of tens to hundreds of paraffin-embedded tissue cores (0.6 to 2 mm in diameter) combined into typically multiple recipient paraffin blocks (Braunschweig et al., 2004). Sectioned slides can then be analyzed like an ordinary tissue section to examine protein staining by immunohistochemistry (IHC), DNA aberrations by fluorescence in situ hybridization (FISH), or mRNA expression levels by mRNA in situ hybridization (Giltinan and Rimm, 2004).

The advantages of this technique to study protein expression are multiple. A TMA represents a high-throughput proteomic analysis that maintains histologic and cytologic
information (Hewitt, 2004). It also allows a large number of tissues, as well as cell lines and control tissues, to be subjected to identical experimental conditions in a short time period (Camp et al., 2008). Furthermore, IHC on TMAs can take advantage of previously collected large tissue archives and requires only a very small sample (Giltanne and Rimm, 2004).

Tissue microarray technology is particularly useful for the discovery and evaluation of cancer biomarkers that aid in diagnosis, prognostication, and prediction of treatment response (Camp et al., 2008). Over the past 15 years, large numbers of human tumours have been profiled on TMAs and much of these data are available on public web pages (Uhlen et al., 2010). In recent years, TMAs have also been used to study canine neoplasms, including osteosarcoma (Khanna et al., 2002), lymphoma (Keller et al., 2007), hemangiosarcoma and hemangiomas (Sabattini and Bettini, 2009), gliomas (Higgins et al., 2010), prostatic carcinoma (Fonseca-Alves et al., 2013; Rodrigues et al., 2011), central nervous system tumours (Wohlsein et al., 2012), and melanomas (Simpson et al., 2014). Additionally, a small-scale TMA (42 cases) was used to investigate KIT protein staining patterns in canine MCTs by immunofluorescence, but found no significant relationships between immunofluorescence, c-KIT mutations and KIT protein localization (Webster et al., 2006).

Validation

The most frequent concern of TMA technology is whether a small tissue core accurately represents the entire tissue section (Giltanne and Rimm, 2004). Several studies have compared biomarker staining in TMA cores compared to large sections. One validation study using human breast carcinoma found that two 0.6 mm cores were representative of tumour antigen expression in more than 95% of cases (Camp et al., 2000). Similarly, in a study of Hodgkin lymphoma, two 0.6 mm cores had a concordance of 93.8% with whole section analysis (Garcia et al., 2003). Another early TMA study examining estrogen and progesterone receptor expression in human breast cancer
found that a single sample 0.6 mm core was sufficient to identify statistically significant associations between expression and clinical outcome (Torhorst et al., 2001).

Another concern with IHC studies in general, is whether there is loss of antigen staining after prolonged storage of blocks (Camp et al., 2000). One study examined antigen immunoreactivity in tissue that dated back more than 60 years (Camp et al., 2000). Although antigen staining could not be compared to when the tissue was collected, as many of the samples were collected prior to the development of IHC, the authors found that many proteins showed successful antigen retrieval and immunoreactivity from samples stored for up to 60 years.

**Tissue microarray scoring**

Tissue microarrays can be scored manually or with an automated analysis. Given the large numbers of cores to be analyzed, one clear advantage of the automated analysis is an extremely rapid analysis that is consistent between every core. Another advantage is the ability to distinguish subtle differences in staining intensity using a continuous scale, which can be difficult for the human eye (Camp et al., 2002). Studies comparing quantitative automated analysis to manual scoring of IHC have found that the automated scoring matched or exceeded the results of conventional manual scoring (Wang et al., 2001). TMAs are frequently quantified by calculating an H-score, which is derived from multiplying the intensity of IHC staining and the percentage of immunopositive cells (Batistatou et al., 2013).

**Study rationale and research proposal**

Taken all together, it is evident that although histological grading is useful for prognostication of canine MCTs, it cannot predict the biological behaviour of each tumour, which can vary from benign to rapidly fatal. Evaluation of biomarkers is an exciting and promising avenue to improve our ability to predict local recurrence, metastasis, survival, and response to
therapy. Furthermore, biomarker characterization of a mutant KIT-driven neoplasm may help in the understanding and treatment of other KIT-driven neoplasms in both dogs and people.

I hypothesize that biomarkers that reflect molecular pathways that are significant in canine MCT biology will correlate with patient outcome, which will help guide future therapy. The specific aims to test this hypothesis are as follows:

1) To construct a TMA of canine cutaneous MCTs with known clinical outcome
2) To analyze biomarker staining profiles in various subsets of the tumours (e.g. dermal vs. subcutaneous, low-grade vs. high-grade)
3) To correlate staining parameters for the biomarkers (all together and between subsets) with patient outcome

A TMA of canine MCTs will provide us with a high-throughput staining profile platform to identify and characterize biomarkers representative of signaling pathways that are critical in MCT biology. This work is significant as it will not only have an immediate impact in improving prognostication but will also lead the way for future mechanistic studies and allow us to further characterize biological differences between dermal and subcutaneous MCTs. Elucidating the role of these pathways in canine MCTs and their interactions with current therapies will enable veterinarians to select personalized targeted treatments and improve patient survival. Furthermore, this work will have broader implications in improving our understanding of other cancers, both canine and human, driven by these pathways.
Chapter 2: Canine Mast Cell Tumour Tissue Microarray Design and Construction with General Prognostic Information on Included Cases

Introduction

The behavior of certain canine mast cell tumours (MCTs) cannot accurately be predicted using current prognostic indicators such as grade or mitotic count. Although grade I and grade III tumours based on the Patnaik grading scheme are generally expected to have an excellent and poor prognosis, respectively, the prognosis for grade II tumours is difficult to predict (Patnaik et al., 1984). Additionally, although high-grade tumours based on the Kiupel grading scheme typically have a poor prognosis, there is a small percentage of low-grade tumours that will recur and/or metastasize, and also possibly lead to MCT-related death of the dog (Kiupel et al., 2011). A prognostication system with higher accuracy is needed, especially as MCTs are one of the most common cutaneous neoplasms in dogs. The use of a tissue microarray (TMA) enables high-throughput immunohistochemistry (IHC) on a large number of tumours at one time, making it a valuable method for prognostic biomarker discovery in cancer studies.

A TMA typically consists of one or more paraffin blocks that each has tens to hundreds of formalin-fixed paraffin-embedded (FFPE) tissue cores. Performing IHC using TMAs is advantageous compared to IHC on conventional full-thickness sections due to simultaneous and consistent antigen retrieval, washing time, antibody concentration, chromogen application, and counter staining on a large number of cores. This ensures consistency in immunostaining between samples, and saves time and resources compared to conventional full-thickness sections. It also allows for stored FFPE samples to be utilized, as the tissues retain antigenicity for years (Camp et al., 2000). In addition, the inclusion of control tissue cores on each TMA block allows for multiple positive and negative internal controls.
One critique of TMAs is whether IHC on small (typically 0.6 mm to 2.0 mm in diameter) cores represent the tumour as a whole. In order to validate the use of TMAs, antigen staining from a large section of the tumour must be compared to antigen staining from 0.6 mm core(s). Using this approach, TMAs have been validated for numerous human cancers, including breast carcinoma (Camp et al., 2000; Torhorst et al., 2001), fibroblastic tumours (Hoos et al., 2001), bladder cancer (Nocito et al., 2001), pulmonary carcinomas (Leversha et al., 2003), and ovarian carcinoma (Rosen et al., 2004), as well as in central nervous system (Wohlsein et al., 2012) and lymphoma tumours (Keller et al., 2007) in the dog.

The objective for this chapter was to design and construct a canine cutaneous MCT TMA for use in MCT prognostic biomarker discovery.

**Materials and Methods**

**Case selection**

Paraffin embedded tissue blocks and haematoxylin and eosin (H&E) slides were collected from the Animal Health Laboratory (AHL) (Ontario Veterinary College, University of Guelph, Guelph, Ontario), Antech Diagnostics Canada (Mississauga, Ontario), and Yager-Best Histovet, Histological and Cytological Services (Guelph, Ontario). The cases from AHL were collected by searching the database for “mastocytoma” (whether the grade was included in the diagnosis code or not, which includes codes E-906, E9061, E9062, and E-9063), in canines, and only cases that were submitted by the Ontario Veterinary College Teaching Hospital (OVCTH) were selected. Of the 104 cases from the OVCTH, the majority were from 2008-2015, with three cases from 2017; the Antech cases were from 2014 (30 cases); and the Yager-Best cases were from 2002-2006 (55 cases). In total, 244 paraffin blocks from 189 dogs were included in the TMA.
Clinical and outcome data

The tissue blocks and medical records for the Yager-Best cases had been collected for a previous study (Thompson et al., 2011a). For the OVCTH cases, the medical records were reviewed to obtain the breed, sex, date of diagnosis, tumour site, details of previous cancer including MCTs, adjunctive treatment protocols, metastatic disease status, date of euthanasia/death, and cause of death. If full outcome data were not available from the medical record, the referring clinic was contacted directly in an attempt to obtain these data.

The date of diagnosis was defined as the date of MCT surgical excision. Disease-free interval (DFI) was defined as the number of days from the date of diagnosis to confirmation (histology or fine needle aspiration) or suspicion (clinical signs) of local recurrence of the MCT or metastasis (lymph node metastasis or disseminated MCT disease in internal organs). Local recurrence was defined as regrowth at the site of surgical excision. Survival time (ST) was defined as the number of days from the date of diagnosis to euthanasia/death or to the date of the last follow-up. The cause of death was recorded as being MCT-related (for example, euthanasia due to local recurrence affecting quality of life or widespread metastases) or unrelated to MCT.

Statistical analyses

Kaplan-Meier functions and plots for DFI and ST were constructed with R statistical programming language (3.5.0) using the “survival” and “survminer” packages, and the logrank test was used to compare functions. Dogs lost to follow-up were censored during statistical analysis. Cox proportional hazard ratios were calculated using the Cox proportional hazard regression model in R, and the Wald test was used to compare hazard ratios in both the univariable and multivariable analyses. Differences were considered significant if $p < 0.05$. 

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**TMA design and construction**

The original histology report was reviewed for all cases for which it was available. For all tumours, H&E stained full thickness sections were examined and areas for TMA coring, type of MCT, Patnaik and Kiupel grade, mitotic count, and KIT staining pattern were determined by a single pathologist (BK). Cases were excluded from the TMA if the neoplastic area on the slide was less than approximately 10 mm$^2$. Three cores were taken from each tumour, unless the tumour was less than approximately 25 mm$^2$, in which case one or two cores were taken. The cases were split across two recipient blocks that each contained 392 positions (Figure 2.1). One corner of each block lacked cores for proper orientation in the event that the section was flipped or rotated during processing. Cores from the same paraffin blocks of normal tissue (canine adrenal gland, cerebellum, cerebrum, kidney, liver, lung, lymph node, mammary gland, ovary, pancreas, pituitary, prostate, salivary gland, skeletal muscle, skin, testis, and uterus) were included on both blocks to act as internal control tissue. All three cores of a single tumour were included on the same block and were randomly assigned to the positions within each block.

**Figure 2.1** Mast cell tumour tissue microarray (TMA) map. Empty boxes represent areas for tissue cores. Shaded boxes represent areas devoid of cores to create asymmetry.
The TMA was constructed with a Pathology Devices TMArrayer™ (Pathology Devices, Westminster, MD, USA). Tissue cores of 0.6 mm in diameter were transferred from donor FFPE blocks to a recipient block. Then, a glass slide was placed on top of the block and the block was incubated at 55 °C for 10 minutes to bind the cores to the surrounding paraffin in the recipient block. The glass slide was kept on the block to prevent desiccation of the cores until the time of sectioning. The TMA blocks were cut into 4 µm thick sections and mounted onto positively charged Superfrost Plus slides (Fisher Scientific, Hampton, NH, USA).

**Immunohistochemistry**

The differences in the IHC steps for each primary antibody that were run manually are listed in Table 2.1. IHC was run within 2 weeks of the slides being sectioned. The general protocol for the antibodies that were manually run was as follows. Unstained tissue sections were baked in the oven overnight at 37 °C. The sections were deparaffinised in xylene and rehydrated. Heat based antigen retrieval was performed using a Biocare Medical Decloaking Chamber NxGen Model: DC2012 (Biocare Medical, Concord, CA, USA) by incubating the sections in their respective buffers at 110 °C for 5 minutes and then allowed to cool to 80 °C. The sections were then incubated with DAKO Peroxidase Blocking Reagent (DAKO Corporation, Carpinteria, CA, USA) for 5 minutes at room temperature, and then incubated with primary antibody overnight at 4 °C. The next day, the sections were incubated with DAKO Envision secondary antibody (DAKO Corporation, Carpinteria, CA, USA) (anti-mouse and anti-rabbit, for mouse and rabbit primary antibodies, respectively) for 30 minutes at room temperature. After washing with TBST, the sections were incubated with 3-3’-diaminobenzidine (DAB) chromogen for 10 minutes. The sections were counterstained with Harris modified hematoxylin for 5 minutes. The slides were then dehydrated, and cover slipped with Cytoseal 60 mounting medium.
Table 2.1 Antibody details for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Species</th>
<th>Company</th>
<th>Product#</th>
<th>Primary Ab dilution</th>
<th>Antigen retrieval buffer</th>
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<tbody>
<tr>
<td>Beclin-1</td>
<td>mAb</td>
<td>Mouse</td>
<td>LSbio</td>
<td>LS-C122820</td>
<td>1:300</td>
<td>Citrate pH6</td>
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<td>C-CBL</td>
<td>pAb</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>2747</td>
<td>1:100</td>
<td>Citrate pH6</td>
</tr>
<tr>
<td>CD117</td>
<td>pAb</td>
<td>Rabbit</td>
<td>Dako</td>
<td>A4502</td>
<td>1:200</td>
<td>Citrate pH6</td>
</tr>
<tr>
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<td>mAb</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>5169</td>
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<td>EDTA pH8</td>
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<tr>
<td>Mouse IgG2a</td>
<td>-</td>
<td>Mouse</td>
<td>Cell Signaling</td>
<td>61855S</td>
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<tr>
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<td>-</td>
<td>Rabbit</td>
<td>Jackson</td>
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<td>Cell Signaling</td>
<td>95005</td>
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<td>Citrate pH6</td>
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</table>

Ab, antibody; mAb, monoclonal antibody; pAb, polyclonal antibody

For each antibody, positive control tissues included in the TMA block were evaluated to confirm positive staining. The positive controls were as follows; Purkinje cells in cerebellum for KIT (Riva et al., 2005), biliary and renal tubular epithelium for beclin-1 (Li et al., 2010; Wang et al., 2017), and Sertoli cells in testis for c-CBL (Da Silva et al., 2017). The beclin-1 and C-CBL antibodies have been verified in canine tissues by Western blot (Da Silva et al., 2017; Schott et al., 2018). A negative isotype control for each type of primary antibody was also run on the TMA. The negative control antibodies included mouse IgG2a (control for mouse monoclonal IgG2a antibodies), rabbit IgG whole molecule (control for rabbit polyclonal antibodies), and rabbit monoclonal IgG isotype control (control for rabbit monoclonal antibodies). The details for the negative control antibodies are also listed in Table 2.1.

Image analysis

The slides were scanned using the Leica SCN400 Slide Scanner automated digital image system (Leica Microsystems, Wetzlar, Germany) by the Digital Histology Shared Resource at Vanderbilt University Medical Center, Nashville, Tennessee. The whole slide images were scanned at 20X magnification at a resolution of 0.5 µm/pixel. The tissue cores were mapped using Ariol Review software within the Digital Image Hub (DIH) (ebcdih.mc.vanderbilt.edu/dih).

The tissue cores were analyzed using the Tissue IA Optimiser program available within the DIH. The default DAB and Fast Red colour definition settings were used for DAB and alkaline
phosphatase red, respectively. Algorithm settings were optimized for the Measured Stained Cells Algorithm. The algorithm settings that were used are listed in Table 2.2.

Table 2.2 Automated image analysis software settings.

<table>
<thead>
<tr>
<th>Input parameters</th>
<th>Settings</th>
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</thead>
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<td>Measurement units</td>
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<td>Tissue threshold</td>
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</tr>
<tr>
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<tr>
<td>Strength of nuclear counterstaining</td>
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</tr>
<tr>
<td>Nuclear area low/high threshold</td>
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<tr>
<td>Nuclei per window low/high threshold</td>
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<tr>
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</tr>
<tr>
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<td>75</td>
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<tr>
<td>Strong/moderate/weak cytoplasmic staining intensity cutoff</td>
<td>AD/AD</td>
</tr>
</tbody>
</table>

AD, antibody-dependent

Results

Clinical data

A summary of the clinical data is listed in Table A.1 in the Appendix. The breed was known for 183 dogs; in which 45 different pure breeds were represented, accounting for 150 of the dogs, with the remaining 33 cases affecting mixed breed dogs. The five most common breeds affected included Labrador Retriever (48 dogs), Boxer (18 dogs), Golden Retriever (10 dogs), Pug (7 dogs), and Boston Terrier (5 dogs). The remainder of the purebreds accounted for less than 4 cases each.

The sex was known for 183 dogs. The majority of the dogs were neutered, with 97 spayed females and 71 neutered males, with the remainder composed of 7 intact females and 8 intact males. The age at MCT diagnosis was known in 183 cases. The average age at diagnosis was 7.7 years of age.
The TMA consisted of 244 different tumour samples from 189 dogs. The vast majority of the samples were biopsies (236 samples), with 8 other samples coming from post-mortem sampling. Most of the samples were primary tumours (213 samples), along with 14 recurrences of tumours, and 17 metastases. Of the 17 metastatic samples, 13 were from lymph nodes, and the remainder were from skin, abdominal wall, and lung. The majority of tumour samples were cutaneous MCTs, with 110 dermal MCTs and 106 subcutaneous MCTs. The location of the remainder of the MCTs were classified as deep/intramuscular, mucocutaneous, penis, and nasal planum.

Of the 216 cutaneous (dermal and subcutaneous) MCTs, the location was known for 202 tumours. There were 74 tumours (37%) on the extremities, 58 (29%) on the thorax, 32 (16%) on the abdomen, 18 (9%) on the head, 15 (7%) in the inguinal/peritoneal area, and 5 (2%) on the neck.

**Outcome data**

Survival data was available for 143 of the 189 dogs. The date of death was known in 74 of these dogs, and in the other 69 cases, the dog was still alive at the last confirmed follow-up date. Of the 74 dogs whose date of death was known, the cause of death could be attributed to MCT-related disease in 41 of the dogs. The DFI was known in 146 dogs, with 88 dogs having no local recurrence of the MCT or metastases at the last known follow-up, and 58 dogs having either local recurrence or metastases (lymph node or disseminated).

Some dogs received adjunctive therapy after surgical removal of the MCT. This treatment consisted of one or a combination of: intravenous chemotherapy (vinblastine, usually in combination with oral prednisone), radiation (palliative or curative), or oral toceranib phosphate (Palladia®). Of the 189 dogs, 44 were recorded to have pursued additional treatment after surgery.
**Mast cell tumour grades**

Of the 110 dermal MCTs, 29 (26%) were classified according to the Patnaik grading scheme (Patnaik et al., 1984) as grade I tumours, 65 (59%) as grade II tumours, and 16 (15%) as grade III tumours. Of these tumours, 45 of them were primary tumours not treated with adjunctive therapy for which outcome data was known; 17 grade I tumours, 24 grade II tumours, and 4 grade III tumours. The disease-specific median survival time (MST) was not reached for grade I tumours, was 1649 days for grade II tumours (95% CI=1530-NA), and was 119 days for grade III tumours (95% CI=36-NA) (Figure 2.2A). The differences in both the survival and disease-free curves were statistically significant (p<0.001) (Figure 2.2).

![Figure 2.2 Kaplan-Meier survival curves for non-AT treated dermal MCTs stratified by Patnaik grade.](image)

(A) shows survival time and (B) shows disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour. *global p<0.05

Of the 216 cutaneous MCTs, 161 (75%) were classified as low-grade MCTs and 55 (25%) were classified as high-grade MCTs according to the Kiupel grading scheme (Kiupel et al., 2011). Of these tumours, 116 were primary untreated tumours for which outcome data was known. The MST was not reached in low-grade tumours and was 211 days in high-grade tumours (95% CI=144-NA) (Figure 2.3A). The difference in survival between the two groups was significant (p<0.0001), and dogs with a high-grade tumour were significantly more likely to die of MCT-related disease than dogs with a low-grade tumour (hazard ratio (HR)=7.3, p<0.0001). The
difference in DFI between low- and high-grade tumours was also statistically significant (p<0.0001), and dogs with a high-grade tumour were significantly more likely to have local recurrence or metastases than dogs with a low-grade tumour (HR=5.6, p<0.0001) (Figure 2.3B).

![Kaplan-Meier survival curves for non-AT treated cutaneous MCTs stratified by Kiupel grade.](image)

**Figure 2.3** Kaplan-Meier survival curves for non-AT treated cutaneous MCTs stratified by Kiupel grade. (A) shows survival time and (B) shows disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05

Of the 106 subcutaneous MCTs, 26 (25%) had a mitotic count of 5 or greater in ten high power 400X fields (area of 2.37 mm²) (high mitotic count), and 80 (75%) had a mitotic count of 4 or less in ten high power 400X fields (low mitotic count). Of these tumours, 71 were primary untreated tumours for which outcome data was known. The MST was not reached in tumours with a low mitotic count and was 442 days in those with a high mitotic count (95% CI=151-NA) (Figure 2.4A). The difference in survival between the two groups was significant (p<0.0001), and dogs with a high mitotic count were significantly more likely to die of MCT-related disease than dogs with a low mitotic count (HR=5.3, p=0.0006). The difference in DFI between low and high mitotic count was statistically significant (p=0.0001), and dogs with a high mitotic count were significantly more likely to have local recurrence or metastases than dogs with a low mitotic count (HR=4.4, p=0.0004) (Figure 2.4B).

Similar results were obtained when comparing low versus high mitotic count for primary untreated dermal tumours. The MST was not reached in tumours with a low mitotic count and was
140 days in those with a high mitotic count (95% CI=98-NA) (Figure 2.4C). The difference in survival between the two groups was significant (p<0.0001), and dogs with a high mitotic count were significantly more likely to die of MCT-related disease than dogs with a low mitotic count (HR=32.1, p=0.002). The difference in DFI between low and high mitotic count was statistically significant (p<0.0001), and dogs with a high mitotic count were significantly more likely to have local recurrence or metastases than dogs with a low mitotic count (HR=10.0, p=0.005) (Figure 2.4D).

**Figure 2.4** Kaplan-Meier survival curves for non-AT treated MCTs stratified by mitotic count. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MC, mitotic count; MCT, mast cell tumour; SC, subcutaneous. *p<0.05
Treatment effects

After surgical removal of the MCTs, adjunct therapy of one or a combination of chemotherapy, radiation, or toceranib phosphate, was pursued in some cases. The MST and DFI of primary cutaneous MCTs were compared between those that received adjunct treatment (one or a combination of chemotherapy, radiation and toceranib) and those that did not (Figure 2.5A,B). Treated tumours had significantly reduced MST and DFI compared to untreated tumours. The outcome of treated versus untreated primary cutaneous MCTs in only the Kiupel high-grade tumours was also examined (Figure 2.5C,D). In high-grade MCTs, there was no significant difference in MST or DFI between those that were treated and those that were not.

Figure 2.5 Kaplan-Meier survival curves for all and Kiupel high-grade cutaneous MCTs stratified by AT status. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05
**Tissue microarray tissue sampling quality**

All tissue spots were evaluated manually to ensure that they accurately represented the targeted sample region, and for quality of the spot. An ideal tissue spot was complete and composed almost entirely of neoplastic mast cells (Figure 2.6A,B). Some cores had partial to complete core loss (Figure 2.6C), while others had folding of the tissue (Figure 2.6D). Some tissue spots had variable amounts of dense connective tissue (collagen) (Figure 2.6E), while other MCTs had neoplastic cells that were separated by edema fluid (Figure 2.6F).

![Figure 2.6 Examples of tissue spots of variable quality and composition.](image)

Tissue spots were excluded from the automated analysis if they were absent or markedly altered (e.g. folded, largely fragmented, *etc.*). Approximately 25 complete tissue spots were manually examined, and the tissue area of each tissue spot was calculated and averaged (approximately 320,000 µm²). The minimum tissue area was therefore defined as the equivalent to approximately 30% of this average area of a complete tissue spot, or 100,000 µm². As part of the subsequent image analysis, the area of all tissue spots from a tumour sample were summed and, in
order to be included in the analysis, the summed area was required to be equivalent to or greater than this minimum tissue area.

**Immunolabelling of tissue spots**

The parameters for the automated analysis were optimized for the MCTs (as listed in Table 2.2). Although a small minority (estimated at less than 5%) of neoplastic mast cells were not detected and measured as cells, the vast majority of neoplastic mast cells were correctly identified and analyzed. The strength of the immunopositivity varied with the antibody, and therefore, the cut-off values to determine negative versus weak versus moderate versus strong staining were optimized for each antibody. For example, the cut-off values for beclin-1 were: less than 220 is negative, between 220 and 185 is weak, between 185 and 180 is moderate, and greater than 180 is strong (see Table 2.3 for staining cut-off values for beclin-1 as well as other antibodies).

**Table 2.3 Antibody-dependent automated image analysis software settings.**

<table>
<thead>
<tr>
<th></th>
<th>Beclin-1</th>
<th>C-CBL</th>
<th>Mast cell tryptase</th>
<th>Cytoplasmic KIT</th>
<th>Membranous KIT</th>
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</thead>
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<td>215</td>
<td>220</td>
<td>220</td>
<td>179</td>
</tr>
<tr>
<td>Strong/moderate/ weak cytoplasmic (or membranous) staining intensity cutoff</td>
<td>180</td>
<td>185</td>
<td>195</td>
<td>210</td>
<td>180 185 152 169</td>
</tr>
</tbody>
</table>

Based on the cut-off values, each cell was determined to have negative (blue), weak (yellow), moderate (orange), or strong (red) immunopositivity in the automated analysis (Figure 2.7). The H-score was then determined by the percentage of cells with negative, weak, moderate, or strong immunopositivity. The H-score for total KIT staining level is the sum of the membranous and cytoplasmic H-scores.
Figure 2.7 Immunohistochemistry staining patterns showing negative, low, medium, and strong cellular cytoplasmic immunopositivity of beclin-1. Two examples of MCTs, one with mostly negative/weak cytoplasmic immunopositivity (A,B), and one with mostly moderate/strong cytoplasmic immunopositivity for beclin-1 (C,D). B and D show the labeled cells after running the automated image analysis software. For the labeled cells algorithm, the colours of the cells are as follows: blue = negative; yellow = weak; orange = moderate; red = strong.

If the tissue spot contained acellular areas (e.g. large areas of collagen, small folds, etc.), or contained adnexal structures or large blood vessels, these areas were often flagged as cellular areas, leading to falsely-identified cells that were negatively immunostaining. Therefore, prior to running the analysis, these areas were delineated with labelled annotations to be excluded from the automated digital analysis. Even with some tissue areas requiring a large exclusion area, using the above-mentioned criteria for minimum tissue area, the number of samples that passed the inclusion criteria was relatively high. For example, for beclin-1, 83% of the tumour samples passed the inclusion criteria.

Discussion

In order to develop improved prognostication of canine MCTs, a TMA was constructed that included 244 tumours from 189 dogs. The various MCTs that comprise the TMA represent different types of MCTs (largely subcutaneous and dermal). The majority of the samples were primary occurrence tumours that were surgically excised by excisional biopsy, but included in the
TMA are also recurrences of MCTs and metastases (both ante- and post-mortem samples).

Although the number of recurrences and metastases is relatively low, this will allow for comparisons of biomarker staining between primary, recurrences, and metastases, which is not well studied in canine MCTs.

The MST and DFI were compared for various subgroups of the MCTs. When primary occurrences of dermal MCTs were stratified by Patnaik grade, there was a statistically significant difference in survival time and DFI, with grade III tumours having the shortest MST and DFI, and grade I tumours having the longest survival time and DFI. These outcome data are consistent with the original published grading scheme (Patnaik et al., 1984). However, as has been the critique of the Patnaik grading scheme, the survival and disease-free curves of this study’s group of MCTs illustrate the difficulty in accurately predicting the outcome for grade II tumours, with 40-50% of dogs being alive or disease-free at 5 years after the date of diagnosis. In our study, none of the dogs with Grade I MCTs experienced MCT-related disease or death, and none of the dogs with Grade III MCTs were alive after 1500 days. However, another limitation of the original Patnaik study, is that 2 of 30 dogs with Grade I MCTs had an MCT-related death, and 1 of 17 dogs with Grade III MCTs had a good outcome, with a survival time of at least 1500 days (Patnaik et al., 1984).

Dogs given adjunct therapy had a significantly reduced MST and DFI compared to those who did not have adjunct therapy. This seemingly odd result is likely explained by the fact that there is a bias in which MCTs get treated. There are several factors that play a role in whether adjunctive treatment is given and of which type, including histological grade, surgical margins, location of the MCT, owner’s willingness, presence of comorbidities, and financial constraints. As histological grade is one of the main determinants, a larger percentage of high grade MCTs will be treated than low-grade MCTs, and this likely explains why the dogs treated with adjunctive
treatment have a poorer outcome than all untreated MCTs. When only high-grade MCTs were compared between treated and untreated, there were no significant differences in MST or DFI. The reason for this is unclear, and perhaps suggests that some of the treated high-grade MCTs did not respond well to treatment.

The MST and DFI for primary occurrences of cutaneous MCTs stratified by Kiupel grade was also calculated. Similar to what was originally published (Kiupel et al., 2011), those dogs with low-grade tumours had a significantly increased MST and DFI than those with high-grade tumours. However, this data set also shows that there are a certain small percentage of dogs that are diagnosed with low-grade MCTs that go on to develop local recurrence or metastases and/or die of MCT-related disease. This again highlights the need to develop better prognostic markers for MCTs.

Mitotic count has been significantly associated with prognosis in subcutaneous MCTs (Thompson et al., 2011a; Thompson et al., 2011b). This analysis showed similar results; dogs with subcutaneous tumours with a mitotic count of 5 or greater in 10 high power 400X fields have a significantly increased risk of death due to MCT-related disease and increased risk of local recurrence or metastases. However, it is important to note that 46 of the 71 primary, subcutaneous MCTs not given adjunctive treatment included in this TMA were from the same group of tumours studied in the Thompson et al. 2011a paper. Mitotic count is also known to be a strong predictor of outcome (Elston et al., 2009; Romansik et al., 2007), and the differences in survival and recurrence or metastasis was also significant when taking all cutaneous (dermal and subcutaneous) tumours into consideration.

Tissue spot partial and complete losses were expected during the design and construction of the TMA. For this reason, if the area of the MCT was large enough, three cores were taken from each MCT. Therefore, even with tissue spot loss and exclusion areas, the number of samples that
passed the minimum tissue area was still relatively high and will enable us to compare biomarker staining with outcome data.

The algorithm parameters for the automated image analysis required optimization for analysis of neoplastic mast cells. During optimization, certain limitations of the automated analysis became apparent. For example, acellular areas (e.g. collagen) were measured as negatively immunolabelled cells. In order to address this, areas of exclusion were manually created to avoid automated analysis of these areas.

In conclusion, an MCT TMA was constructed, containing a large sample of primary cutaneous MCTs, as well as a smaller number of recurrences and metastases. The primary cutaneous MCTs covered a range of grade and mitotic activity and were fairly evenly split between dermal and subcutaneous tumours. The automated analysis parameters were optimized for neoplastic mast cells as well as for specific antibodies, areas that necessitated exclusion were identified and annotated, and a minimum tissue area was defined. Outcome data was collected for as many animals as possible, and the next step is to utilize this TMA in combination with the outcome data for novel prognostic biomarker discovery.
Chapter 3: Investigation of the Prognostic Biomarkers KIT, c-CBL, and Tryptase in Tissue Microarrays of Canine Mast Cell Tumours

Introduction

Canine mast cell tumours (MCTs) are common skin tumours of dogs whose behaviour is often difficult to predict. Novel biomarkers would be useful in helping to prognosticate tumours and to guide adjunctive therapy. A small number of biomarkers have been examined in canine MCTs. The expression of VEGFR-1 and -2 has been evaluated in canine neoplastic mast cells from cell lines (Rebuzzi et al., 2007; Sekis et al., 2009) and from a small number of MCTs (Da Silva et al., 2017; Thompson et al., 2015). Overall, high VEGFR2 and phosphorylated VEGFR2 expression in the neoplastic cells was associated with decreased survival, increased metastasis and local recurrence (Da Silva et al., 2017; Thompson et al., 2015). Examination of PDGFRα/β revealed expression of PDGFRβ in a subset of tumours, but there was no significant correlation with clinical outcome (Thompson et al., 2015).

In non-neoplastic mast cells, the KIT protein is typically located within the cellular membrane. However, in some neoplastic mast cells, there is aberrant localization of Kit within the cytoplasm. Three different KIT staining patterns can be observed in canine MCT cells; pattern I is membranous; pattern II is focal or stippled cytoplasmic; and pattern III is diffuse cytoplasmic. Cytoplasmic staining has been associated with activating c-KIT mutations (Webster et al., 2006), and reduced DFI and survival (Kiupel et al., 2004; Thompson et al., 2015; Webster et al., 2007).

Tryptase is one of the most abundant proteinases that is produced and stored in mast cells. Canine MCTs that are poorly differentiated typically have fewer positively staining granules, both with hematoxylin and eosin, as well as metachromatic toluidine blue-stained granules (Simoes and Schoning, 1994). As this decreased staining is thought to be related to decreased production or storage of the mast cell produced compounds, it is possible that decreased tryptase may be
correlated to less differentiated MCTs and poorer prognoses. An IHC analysis of tryptase in canine neoplastic mast cells identified three different patterns. Tryptase-staining pattern I was categorized as diffuse cytoplasmic immunopositivity, tryptase-staining pattern II was a moderate amount of cytoplasmic stippling, and tryptase-staining pattern III was weak cytoplasmic stippling (Kiupel et al., 2004). However, the tryptase-staining patterns were not significantly associated with disease-free survival or local recurrence (Kiupel et al., 2004).

C-Casitas B-lineage lymphoma (c-CBL) is a member of a highly conserved family of E3 ubiquitin protein ligases which mono-ubiquitinate many proteins, including receptor tyrosine kinases (RTKs) such as KIT, VEGFR2, and PDGFR. Ubiquitination of these proteins targets them for degradation within the proteasome. Mutations in CBL result in strong surface expression of KIT in human acute myeloid leukemia cells (Makishima et al., 2012). Mutant retrovirally transduced CBL genes also resulted in a generalized mastocytosis in mice (Bandi et al., 2009). Interestingly, a recent study has shown that neoplastic mast cell expression of c-CBL in canine MCTs was associated with reduced disease-free survival (Da Silva et al., 2017).

Given the role of c-CBL in RTK biology, as well as the relationship between c-CBL and clinical outcome, the goal of this study was to further characterize staining of these biomarkers on a large number of canine MCTs in a TMA. Although mast cell tryptase staining-patterns were not significantly associated with survival outcome (Kiupel et al., 2004), another goal was to investigate whether cytoplasmic staining levels, regardless of the pattern of staining, would provide prognostic information in canine MCTs. To this end, a canine MCT TMA was constructed (Chapter 2) with known clinical outcome to investigate potential prognostic markers.
Materials and Methods

Case selection

Cases were included in the analysis if they were excisional biopsies of dermal or subcutaneous MCTs, either primary occurrences or reoccurrences. For the analysis of the treated recurrent tumours, there was one case whose primary MCT was also included in the TMA; all other recurrences did not have the paired primary MCT included in the TMA. Cases of excisional biopsies were included regardless of the length of surgical margins as measured by radial sectioning and histopathology.

TMA construction and immunohistochemistry

TMA construction and immunohistochemical methods are described in Chapter 2.

KIT staining patterns

As the automated digital image system is not designed to separate focal/stippled cytoplasmic from diffuse cytoplasmic staining, and automated analyses have not been previously used for KIT staining patterns, the tissue spots from the full TMA were also manually reviewed by a single pathologist (BK), and the KIT staining pattern was determined. The tissue spots were evaluated knowing the case number, but having no knowledge of the outcome data. In MCTs with neoplastic cells showing a mixture of two or three patterns, the staining pattern was determined by the pattern that was present in the greatest percentage of cells. In order to make an accurate determination of the KIT staining pattern, at least approximately 100 neoplastic mast cells were required to be included in the manual analysis.

Automated analysis

The automated analyses, including annotation of acellular regions, use of the previously defined minimum tissue area, and the use of the algorithm parameters, were performed in an
identical manner to those described in Chapter 2. H-score cut-offs of 200, 8, and 50, were used to stratify low- versus high-expressing KIT, c-CBL, and mast cell tryptase tumours, respectively.

Results

KIT staining patterns

There were 96 cases of cutaneous MCTs not treated with adjunctive therapy (untreated) for which both outcome data was known, and the KIT staining pattern could be determined from the TMA. The tumours were fairly evenly split between the three different patterns, with 38 pattern I, 32 pattern II, and 26 pattern III tumours. The disease-specific MST was not reached in patterns I and II, and was 1710 days for pattern III tumours (95% CI=673-NA) (Figure 3.1A). The difference in survival curves was statistically significant (p=0.015). There was a significant difference between the hazard ratio of pattern III compared to I (HR=3.0, p=0.03) and III compared to II (HR=3.9, p=0.02), but not between patterns I and II. The difference in DFI comparing the three patterns was also statistically significant (p=0.03) (Figure 3.1B), and there was a significant difference between pattern III compared to I (HR=2.6, p=0.04) and pattern III compared to II (HR=3.3, p=0.03), but not between patterns I and II.

Figure 3.1 Kaplan-Meier survival curves for non-AT treated cutaneous MCTs stratified by KIT pattern. (A) shows survival time and (B) shows disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; KP, KIT pattern; MCT, mast cell tumour; SC, subcutaneous. *global p<0.05
**KIT staining levels**

The automated analysis calculates a separate membranous and cytoplasmic H-score. Total KIT staining levels were analyzed by summing the membranous and cytoplasmic H-score. In primary, untreated and treated, cutaneous MCTs, the total KIT staining levels were highest in pattern I (mean H-score=282, n=64), and lower levels were found in both pattern II (mean H-score=185, n=48), and pattern III (mean H-score=209, n=35) (Figure 3.2). There was an overall significant difference in mean H-scores, a significant difference between patterns I and II (p<0.001) and patterns I and III (p=0.006), but not between patterns II and III (p=0.6) as calculated with a Tukey post hoc test.

![Figure 3.2 Violin plot of average KIT H-scores by KIT staining patterns.](image)

The KIT total staining levels were also compared between different categories of tumours. The KIT staining levels were highest in recurrent tumours (mean H-score=267, n=12), lower in primary tumours (mean H-score=230, n=155), and lowest in metastatic tumours (mean H-score=208, n=14) (Figure 3.3). There were no significant overall differences in mean H-scores as calculated with an ANOVA, nor between pairs of means in a Tukey post hoc test.
Figure 3.3 Violin plot of average KIT H-scores in primary, recurrent and metastatic canine cutaneous mast cell tumours. The overlying boxplot displays the median and quartiles.

To investigate whether mast cell KIT staining could be a potential prognostic marker, survival and disease-free curves were compared between low and high-expressing KIT MCTs. The differences in survival and disease curves between the two groups was not significant (Figure 3.4).

Figure 3.4 Kaplan-Meier survival curves for non-AT treated MCTs stratified by KIT. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

Mast cell tryptase staining in primary, recurrent and metastatic tumours

The staining of mast cell tryptase was also investigated as a potential biomarker. The immunopositivity of mast cell tryptase was almost exclusively cytoplasmic with a pattern that varied from weak, diffuse to strong, diffuse immunopositivity. The mast cell tryptase staining
levels were lowest in primary tumours (mean H-score=78, n=169), marginally higher in metastases (mean H-score=82, n=14), and highest in recurrences (mean H-score=98, n=12) (Figure 3.5). There were no significant overall differences in means H-scores as calculated with an ANOVA, nor between pairs of means in a Tukey post hoc test.

![Tryptase H-scores in dermal and subq](image)

Figure 3.5 Violin plot of average mast cell tryptase H-scores in primary, recurrent and metastatic canine cutaneous mast cell tumours. The overlying boxplot displays the median and quartiles.

Mast cell tryptase staining in untreated tumours

Although there were no differences in mast cell tryptase H-scores between primary, recurrent, and metastatic cutaneous MCTs, an investigation into whether mast cell tryptase staining could be a potential prognostic marker was conducted. Survival and disease-free outcome in all primary untreated cutaneous MCTs were compared, and there was no significant difference in MCT-specific survival or DFI (Figure 3.6A,B). There were also no significant differences when the MCTs were separated into dermal (Figure 3.6C,D) and subcutaneous (Figure 3.6E,F).
Figure 3.6 Kaplan-Meier survival curves for non-AT treated cutaneous MCTs stratified by tryptase. (A,C,E) show survival time and (B,D,F) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

Although there were no differences in MCT-related survival or DFI in all primary untreated tumours, MCTs display a wide variety of behaviour, and it is possible that tryptase staining levels could be prognostic within certain subsets of MCTs. To investigate this possibility, outcome data
stratified by tryptase staining was examined from dogs with cutaneous low- and high-grade MCT, as defined by the Kiupel grading scheme (Figure 3.7). Interestingly, there is a significant difference in the DFI curves in primary, untreated, low-grade cutaneous (dermal and subcutaneous) MCTs, and a Cox proportional hazard analysis calculated a hazard ratio of 3.2 (p=0.051). If there was a method to distinguish those low-grade MCTs that are actually aggressive tumours, this could prove extremely useful in helping clients decide whether to pursue additional treatment after surgical excision of low-grade MCTs.

![Kaplan-Meier survival curves for non-AT treated low- and high-grade cutaneous MCTs stratified by tryptase](image)

Figure 3.7 Kaplan-Meier survival curves for non-AT treated low- and high-grade cutaneous MCTs stratified by tryptase. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05

To investigate this further, outcome data in other subgroups of MCTs, divided between those with better and poorer prognoses was analyzed. In the analysis of KIT staining patterns
(Figure 3.1), there was a significant difference in survival and DFI between patterns III and I, and patterns III and II, but not between patterns I and II. Therefore, the outcome between low and high tryptase in pattern I and II combined, as well as in those tumours with pattern III was compared (Figure 3.8). There was a non-significant decreased time to recurrent or metastatic disease compared to the high tryptase group in the primary, untreated, pattern I/II group (p=0.11).

![Kaplan-Meier survival curves for non-AT treated KIT patterns I/II and III cutaneous MCTs stratified by tryptase.](image)

**Figure 3.8** Kaplan-Meier survival curves for non-AT treated KIT patterns I/II and III cutaneous MCTs stratified by tryptase. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

Next, outcome in dogs with dermal tumours categorized according to the Patnaik grading scheme was examined. Unfortunately, there were no events in either MCT-related survival or disease within the primary, untreated, grade I, dermal MCTs for which a tryptase H-score was available.
In both the grade II and grade III MCTs, there were no differences in MST or DFI between low and high tryptase expressing MCTs (Figure 3.9).
Figure 3. Kaplan-Meier survival curves for non-AT treated Patnaik grade II and III cutaneous MCTs stratified by tryptase. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. The only robust prognostic marker that has been identified specifically for subcutaneous MCTs, is the mitotic count (Thompson et al., 2011a). Therefore, outcome between low and high tryptase expressing subcutaneous MCTs in those with a low mitotic count was also examined (Figure 3.10A,B), and those with a high mitotic count (Figure 3.10C,D). Notably, there is again a significant difference in the DFI of those MCTs with a better prognosis, namely the low mitotic count subcutaneous MCTs; low tryptase expressing ones have a shorter DFI than high tryptase subcutaneous MCTs. A Cox proportional hazard analysis also shows a significant difference (HR=5.1, p=0.039).
Figure 3.10 Kaplan-Meier survival curves for non-AT treated low and high mitotic count subcutaneous MCTs stratified by tryptase. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05

C-CBL staining in primary, recurrent and metastatic tumours

A similar analysis was conducted with c-CBL, another potential prognostic marker of interest. In the MCTs with neoplastic mast cells that showed c-CBL immunopositivity, the pattern varied from weak to moderate, and was exclusively diffuse, cytoplasmic immunoreactivity. The c-CBL staining levels were lowest in recurrent tumours (mean H-score=3.9, n=12), higher in primary tumours (mean H-score=10.0, n=163), and highest in metastases (mean H-score=21.9, n=15) (Figure 3.11). There was a significant difference in means as calculated with an ANOVA (p=0.0496); however, a Tukey post hoc test showed no significant differences in means between primary and recurrence (p=0.58), primary and metastasis (p=0.08), or recurrence and metastasis (p=0.06). The violin plot does show that there are a small number of outliers in the group of primary tumours with high c-CBL staining levels. Given that the metastases also have higher c-CBL staining, it was hypothesized that high c-CBL levels in primary MCTs would have decreased times to MCT-related disease and/or death.

Figure 3.11 Violin plot of average c-CBL H-scores in primary, recurrent and metastatic canine cutaneous mast cell tumours. The overlying boxplot displays the median and quartiles.
The MST and DFI in primary, untreated cutaneous MCTs stratified by c-CBL staining levels was analyzed first. There were no significant differences in MCT-specific MST or DFI with all cutaneous MCTs grouped together (Figure 3.12A,B), or separated into dermal (Figure 3.12C,D) and subcutaneous (Figure 3.12E,F) MCTs.
Figure 3.12 Kaplan-Meier survival curves for non-AT treated cutaneous MCTs stratified by c-CBL. (A,C,E) show survival time and (B,D,F) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

Considering that tryptase appears to be a prognostic marker for those tumours with a good (but not poor) prognosis, outcome between low and high c-CBL expressing MCTs in different categories of MCTs was examined. The MSTs and DFIIs were analyzed in dogs with MCTs stratified by c-CBL staining in dogs; with low- and high-grade cutaneous MCTs, as defined by the Kiupel grading scheme; with KIT staining pattern I/II and III; with grade II and grade III dermal MCTs, as defined by the Patnaik grading scheme; and with low and high mitotic count subcutaneous MCTs. None of these survival curves showed any significant differences (data not shown).

Although the initial objective was to find a novel prognostic marker by analyzing survival data of primary MCTs not treated with adjunctive therapy, MCT samples from dogs that did go on to receive adjunctive treatment after surgical excision was also included in the TMA. Therefore, the dataset could be probed to ask whether c-CBL was a potential predictive marker in canine MCTs. The MST and DFI were analyzed for dogs who had received chemotherapy (vinblastine), curative or palliative radiation, and/or toceranib following excisional biopsy. These analyses were done for primary MCTs, as well as for primary combined with recurrent MCTs (referred to as “All” within this and subsequent figure titles). The small sample size of recurrent MCTs precluded analyses of recurrent MCTs alone. There were no significant differences in MST or DFI in primary treated cutaneous (dermal and subcutaneous) MCTs (Figure 3.13A,B), or primary together with recurrent MCTs (Figure 3.13C,D). In the primary, adjunctive therapy treated cutaneous MCTs, the high c-CBL expressing MCTs showed a non-significant decreased MST compared to the low c-CBL expressing MCTs (Figure 3.13A, p=0.081).
Kaplan-Meier survival curves for AT treated cutaneous MCTs stratified by c-CBL. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

Considering that the overall prognosis is different between subcutaneous MCTs (which tend to have a better prognosis) versus dermal MCTs (Thompson et al., 2011a), c-CBL staining levels were analyzed for predictive value separately in dermal and subcutaneous MCTs. There were no significant differences in MST or DFI in the treated dermal MCTs (Figure 3.14). Interestingly, high c-CBL expressing primary subcutaneous MCTs showed a non-significant decreased MST compared to low c-CBL ones (Figure 3.15A, p=0.057), whereas no difference was observed in DFI (Figure 3.15B). A non-significant decreased survival time was also present for primary combined with recurrent MCTs (Figure 3.15C, p=0.13), but no difference in the DFI
The Cox proportional hazard ratio for primary treated subcutaneous MCTs was 4.6 (p=0.08), and for primary and recurrent treated subcutaneous MCTs was 3.1 (p=0.15).

Figure 3.14 Kaplan-Meier survival curves for AT treated dermal MCTs stratified by c-CBL. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.
Figure 3.15 Kaplan-Meier survival curves for AT treated subcutaneous MCTs stratified by c-CBL. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

The survival functions stratified by c-CBL staining were also compared for treated low- and high-grade cutaneous MCTs, as defined by the Kiupel grading scheme; with grade II dermal MCTs, as defined by the Patnaik grading scheme; low mitotic count subcutaneous MCTs; and low and high mitotic count cutaneous (dermal and subcutaneous) MCTs. None of these survival curves showed any significant differences (data not shown). There were not enough cases of high mitotic count subcutaneous MCTs to compare survival functions.

Discussion

In this study, a TMA of canine untreated and treated with adjunctive therapy, dermal and subcutaneous, MCTs was constructed and IHC for KIT, mast cell tryptase and c-CBL was
performed. The MCTs were split in low- and high-expressing KIT, mast cell tryptase and c-CBL groups, and survival functions were compared. Total KIT staining levels were not prognostic. This result is consistent with a study that analyzed a smaller TMA of MCTs that showed no relationship between KIT staining levels and two established prognostic factors, namely, the presence of c-KIT mutations and KIT staining pattern (Webster et al., 2006).

There was a significant difference in DFI in primary, no adjunctive therapy, low-grade cutaneous MCTs stratified by tryptase, as well as in DFI in primary, no adjunctive therapy, low mitotic count subcutaneous MCTs, but no significant differences in adjunctive treated tumours. In both low-grade combined dermal and subcutaneous MCTs, and low mitotic count subcutaneous MCTs, those MCTs with low tryptase had decreased time to recurrence and/or metastasis compared to high tryptase MCTs. There was also a non-significant trend of high c-CBL being predictive of poor response to adjunctive treatment as measured by MST in MCTs that were treated with one or a combination of toceranib, vinblastine, or radiation, but no significant differences in no adjunctive therapy treated tumours. The decreased MST in high c-CBL-expressing tumours in treated MCTs was significant in subcutaneous, but not dermal, MCTs when these tumours were analyzed separately.

If tryptase is indeed a prognostic marker for disease recurrence and/or metastasis in low-grade cutaneous MCTs, and low mitotic count subcutaneous MCTs, as our data suggests, this is clinically useful. One of the main limitations of the Kiupel grading scheme, is that up to 15% of low-grade MCTs have aggressive behaviour and will go on to recur or metastasize despite their designation as a low-grade tumour (Kiupel et al., 2011; Stefanello et al., 2015). If low tryptase levels can predict an increased likelihood of these events, it may help direct decisions on whether to pursue adjunctive treatment after surgical removal of these low-grade tumours.
Humans do not develop solitary cutaneous MCTs like dogs, but they do develop pathological proliferations of abnormal mast cells in the skin (cutaneous mastocytosis) or in internal organs (systemic mastocytosis) (Le et al., 2017). Rather than being used as a prognostic marker of mastocytosis, serum tryptase levels instead are used as a criterion for the diagnosis of systemic mastocytosis, and as a marker to assess response to treatment with bone marrow transplantation (Komi et al., 2018). Interestingly, there have been investigations looking at tryptase levels in other types of human cancer, however, these studies have focused on high tryptase non-neoplastic mast cells within non-mast cell tumours. For example, high levels of tryptase positive mast cells are predictive of poorer outcome in patients with colorectal liver metastases following hepatectomy (Suzuki et al., 2015). Increased mast cell density is also correlated with increased tumour angiogenesis; tryptase has a direct angiogenic effect through stimulating vascular endothelial cell proliferation (Ammendola et al., 2014; de Souza Junior et al., 2015).

In the case of canine MCTs, it is actually low tryptase levels that predict poorer outcome. This is likely related to the fact that tryptase levels in neoplastic mast cells are being measured, rather than non-neoplastic tumour associated mast cells. As tryptase is normally produced in mast cells, lower levels of tryptase are likely related to a lower degree of differentiation (Simoes and Schoning, 1994). And as a general rule for cancers, both human and canine, poorly differentiated tumours tend to behave more aggressively. Indeed, in canine MCTs, those tumours classified as “undifferentiated” based on a high-throughput gene staining analysis had a poorer prognosis than those classified as “differentiated” (Giantin et al., 2014). Therefore, the decreased differentiation of the neoplastic mast cells likely ‘trumps’ any effect (if present at all) of tryptase as a pro-angiogenic and tumour-promoting factor in canine MCTs.

The analysis of c-CBL staining in our TMA showed no prognostic value in c-CBL staining level, but a potential predictive value in primary subcutaneous MCTs. c-CBL staining levels
showed no prognostic information in subcutaneous MCTs in contrast to a report that showed that high c-CBL levels were associated with decreased DFI (Da Silva et al., 2017). There are some differences between the two analyses that could explain this discrepancy. One main difference is in the analysis of the data. Our study used an automated digital analysis of a scanned slide to detect positive immunoreactivity in each cell, whereas the Da Silva et al. analysis used Image J software to determine the percent positively stained area of representative areas of the tumour. Another difference is that in this study, excisional biopsies of MCTs regardless of the surgical margins were included, whereas the Da Silva et al. analysis included only those tumours with neoplastic cells within 1 mm of the margins. Additionally, in our study, not all the dogs eventually developed recurrences (14 of the 57 dogs with analyzed subcutaneous MCTs developed local recurrences), whereas all dogs in the Da Silva et al. analysis did eventually develop local recurrences. Further studies will be needed to investigate this further.

Interestingly, in the Da Silva study (almost all subcutaneous MCTs that were not treated with adjunctive therapy), higher c-CBL is associated with decreased DFI compared to low c-CBL staining MCTs, while in this study (in adjunctive therapy treated subcutaneous MCTs), higher c-CBL is non-significantly associated with decreased survival (p=0.057). This might initially seem surprising, given that MCTs are KIT-driven tumours, and one might expect that high c-CBL would result in increased destruction of the KIT protein and lead to a better outcome. However, in c-CBL has been reported to be upregulated in several human solid tumours (Cristobal et al., 2014; Ito et al., 2004; Jing et al., 2016; Knight et al., 2008; Tan et al., 2010), and high c-CBL levels are associated with poor prognosis in gastric carcinoma (Ito et al., 2004), gliomas (Jing et al., 2016), and prostate cancer (Knight et al., 2008).

The positive role that c-CBL plays in tumourigenesis may be related to the fact that in addition to acting as an E3 ubiquitin ligase, c-CBL is also a multifunctional adaptor protein that
positively regulates various signaling pathways, such as the PI3K/AKT pathway (Liyasova et al., 2015). Therefore, even in KIT-driven tumours such as canine MCTs, it may not be its role on RTK degradation, but rather its adaptor protein role, that promotes tumour progression. Consistent with this hypothesis, a mutant c-CBL protein that lacks ubiquitin ligase activity but maintains its association with SH2-domain-containing signaling proteins, is able to induce alterations in cell morphology that are typically associated with epithelial-mesenchymal transition (Fournier et al., 2000). Additionally, in human gliomas, c-CBL was found to play a positive role in tumour invasion through the upregulation of matrix metalloproteinase 2 (Lee and Tsygankov, 2010). There is also evidence of c-CBL playing a positive role in tumourigenesis through its E3 ligase function. The siRNA-mediated suppression of c-CBL and CBL-B inhibited the invasiveness of breast cancer cells, and these results were thought to relate to their function to ubiquitinate AMAP1, a protein known to play a role in invasion of tumour cells (Nam et al., 2007).

This TMA included both untreated and treated MCTs, enabling us to investigate the potential prognostic and predictive value of two biomarkers, mast cell tryptase and c-CBL. The main limitation of this TMA is that although a large number of overall MCTs were included, the numbers of tumours were lower when categorized into untreated and treated excisional biopsy subsets, and especially when also divided into further subsets based on grade and mitotic count. However, this TMA is an excellent screening tool to identify potential predictive and prognostic markers, and given that statistically significant results were observed, the findings are extremely promising. Another limitation to this study is that this was a retrospective analysis, restricting which MCTs could be included in the TMA (e.g. based on availability of blocks, size of tumour, no standardized treatment protocols for included tumours, etc.), and some of the dogs were lost to follow-up. Further prospective studies with pre-determined inclusion criteria, and extended follow-up data, will help in confirming our results.
In conclusion, this study found that low tryptase staining in neoplastic mast cells was significantly associated with decreased time to local recurrence and/or metastasis in low-grade cutaneous MCTs and low mitotic count subcutaneous MCTs. Additionally, high c-CBL expressing subcutaneous MCTs tended to show poorer response to treatment (as measured by MCT-related survival) compared to low c-CBL expressing subcutaneous MCTs. Having an indication of which low-grade MCTs will go on to recur and/or metastasis could be extremely helpful in directing therapy. Perhaps with adjunctive treatment (that would not normally have been pursued given the diagnosis of a low-grade tumour), the outcome of those low-grade low-tryptase MCTs will improve. Additionally, having a potential predictive factor for response to treatment in subcutaneous MCTs could also help in directing treatment options.
Chapter 4: Beclin-1 is a Novel Predictive Biomarker for Canine Mast Cell Tumours

Introduction

Canine cutaneous mast cell tumours (MCTs) is the most common skin tumour of the dog, representing up to 21% of all cutaneous tumours (Bostock, 1986; Cohen et al., 1974; London and Seguin, 2003; Welle et al., 2008). Cutaneous MCTs arise in either the dermis or within the subcutaneous tissue, at a ratio of approximately 6:1 (Sabattini et al., 2015). The behaviour of cutaneous MCTs can vary widely, with many of the tumours having a favourable prognosis, and fewer MCTs developing local recurrence and/or metastases to the draining lymph node or disseminated throughout the body, which typically includes, but is not limited to, spleen and liver.

The ability to accurately predict the behaviour of a cutaneous MCT is critical in directing patient therapy. Although many factors have been shown to be prognostic, the most widely used prognostic indicators for dermal MCTs are two different grading schemes. The first widely used grading scheme to be developed was the Patnaik grading scheme (Patnaik et al., 1984), which was uniquely developed for dermal MCTs. The second more recently developed grading scheme, which included both dermal and subcutaneous MCTs in its development, is the Kiupel grading scheme (Kiupel et al., 2011). The most useful prognostic indicator for subcutaneous MCTs is the mitotic count (Thompson et al., 2011a).

Although much progress has been made in improving our ability to prognosticate MCTs, the current schemes still have limitations. For example, in the Patnaik grading scheme, the majority of MCTs fall into the grade II category, whose behaviour is quite difficult to predict (Sabattini et al., 2015; Stefanello et al., 2015). In addition, approximately 5-15% of dogs with Kiupel low-grade MCTs (which represented 76-90% of the studied tumours) died or were euthanized due to MCT-related disease (Kiupel et al., 2011; Stefanello et al., 2015).
Efforts to improve prognostication have been made through characterization of the receptor tyrosine kinase (RTK), KIT. Canine normal and neoplastic mast cells typically display one of three distinct KIT staining patterns, namely membranous (pattern I), focal/stippled cytoplasmic (pattern II), and diffuse cytoplasmic (pattern III). Normal mast cells show membranous KIT staining, whereas neoplastic cells can show any one of the three patterns (Webster et al., 2006). Cytoplasmic KIT protein localization (patterns II and III) is significantly associated with increased tumour recurrence and reduced survival (Kiupel et al., 2004; Thompson et al., 2015; Webster et al., 2007), higher histological grade and increased cell proliferation (Gil da Costa et al., 2007) in dermal MCTs, and increased recurrence and metastases in subcutaneous MCTs (Thompson et al., 2015). Although the use of KIT staining pattern is a step forward in prognostication, it still cannot predict the behaviour of every tumour. Given the limitations of the current prognostication systems, we were interested in investigating novel prognostic biomarkers for cutaneous MCTs.

In addition to the development of accurate prognostication for both human and animal cancer, there has also been tremendous effort to develop accurate predictive markers. Predictive markers are imperative in the development of personalized medicine, or choosing specific therapeutics based on the characteristics of an individual’s tumour. The only predictive marker that has been investigated in canine MCTs, is the c-KIT mutational status. However, as MCTs with both mutated and wildtype c-KIT respond to treatment with RTK inhibitors (Hahn et al., 2010; London et al., 2009; Weishaar et al., 2018), it is not a clinically useful predictive marker.

Autophagy is a homeostatic mechanism operating at low basal levels that enables destruction of damaged organelles and proteins. To survive times of adverse microenvironmental conditions, such as nutrient starvation or growth factor depletion, cells can induce autophagy to degrade and recycle cellular components to maintain a source of building blocks (Bialik et al., 2018). The process of autophagy begins with nucleation, whereby multiple proteins assemble to
form a phagophore. Next, the walls of the phagophore elongate, curve around, and join together to form the autophagosome. The final step consists of fusion of the autophagosome with the lysosome and subsequent protein degradation. Cancer cells are also able to exploit the process of autophagy in order to survive the poor conditions in the tumour microenvironment (Ozpolat and Benbrook, 2015). The protein beclin-1 is encoded by the autophagy related gene, BECN1, and plays a key role in the nucleation step of autophagy (Mizushima, 2007). The aim of this study is to investigate beclin-1 staining levels as a prognostic and/or predictive marker in canine dermal and subcutaneous MCTs.

**Materials and Methods**

**Case selection**

Cases were included in the analysis if they were excisional biopsies of dermal or subcutaneous MCTs, either primary occurrences or reoccurrences. For the analysis of the treated recurrent tumours, there was one case whose primary MCT was also included in the TMA; all other recurrences did not have the paired primary MCT included in the TMA. Cases of excisional biopsies were included regardless of the length of surgical margins as measured by radial sectioning and histopathology.

**TMA construction and immunohistochemistry**

TMA construction and immunohistochemical methods are described in Chapter 2.

**Automated analysis**

The automated analyses, including annotation of acellular region, use of the previously defined minimum tissue area, and the use of the algorithm parameters, were performed in an identical manner to those described in Chapter 2. An H-score cut-off of 80 was used to stratify low- versus high-expressing beclin-1 tumours.


**Results**

**High beclin-1 staining is correlated with recurrence and metastasis**

One advantage of using an automated digital analysis, is that a quantitative (rather than categorical) measurement of the amount of protein staining is generated. Therefore, the beclin-1 staining could be analyzed to see if it varied between primary, recurrent, and metastatic canine cutaneous MCTs. The staining of beclin-1 in MCTs was exclusively cytoplasmic, and varied from moderate to strong in intensity, and from granular to diffuse cytoplasmic in pattern. Interestingly, the beclin-1 staining was lowest in primary tumours (mean H-score=73, n=166), higher in recurrences (mean H-score=110, n=12), and highest in metastases (mean H-score=157, n=15) (Figure 4.1). There was a significant difference in means as calculated with an ANOVA (p<0.001), with a Tukey post hoc test showing a significant difference in means between primary and metastasis (p<0.001), but not between primary and recurrence (p=0.07) or recurrence and metastasis (p=0.09).

![Figure 4.1 Violin plot of average beclin-1 H-scores in primary, recurrent and metastatic canine cutaneous mast cell tumours. The overlying boxplot displays the median and quartiles.](image)

**Beclin-1 is not a prognostic biomarker for canine mast cell tumours**

Given that increased beclin-1 levels correlated with increased aggressive behaviour of a tumour, it was hypothesized that beclin-1 staining may be a prognostic marker in canine MCTs. In
order to answer this question, beclin-1 levels in primary, untreated tumours were examined, to determine whether high levels of beclin-1 staining would predict decreased survival and/or decreased time to local recurrence or metastasis. Survival times for MCT-specific deaths and DFI for low versus high beclin-1 staining (as shown in Figure 2.7) in all primary untreated cutaneous MCTs (Figure 4.2A,B) were examined. There was no significant difference in MCT-specific survival or DFI. This result was the same when the MCTs were separated into dermal (Figure 4.2C,D) and subcutaneous (Figure 4.2E,F) MCTs.
Figure 4.2 Kaplan-Meier survival curves for non-AT treated MCTs stratified by beclin-1. (A,C,E) show survival time and (B,D,F) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

Although there were no differences in MCT-related survival or recurrence/metastasis when examining all primary tumours not given adjunctive therapy, the possibility remained that beclin-1 staining levels could be prognostic within certain subsets of MCTs that show different clinical
behaviour. To investigate this possibility, outcome data stratified by beclin-1 staining in several different subsets of the primary untreated MCTs was examined; from dogs with cutaneous low- and high-grade MCTs, as defined by the Kiupel grading scheme; from dogs with cutaneous KIT staining pattern I/II and pattern III MCTs; from dogs with dermal Grade II and III MCTs, as defined by the Patnaik grading scheme (there were no events in those dogs with Grade I MCTs); and from dogs with subcutaneous low and high mitotic count MCTs. No significant differences in the survival or disease-free curves were present (p-values are summarized in Appendix Table A.2). Taking into account the analyses of subsets of cutaneous MCTs, it is clear that staining differences of beclin-1 is not prognostic, even within subsets of tumours.

**Survival curves of mast cell tumours given adjunctive therapy, stratified by beclin-1 staining levels**

Although our initial aim in developing the MCT TMA was to identify a novel prognostic marker utilizing outcome data in dogs with primary MCTs not given adjunctive therapy, the advantage of creating a TMA is that additional samples could be included at minimal extra cost and time. As many of our samples were obtained from a tertiary care clinic with an on-site cancer centre, a number of the MCT samples came from dogs who pursued adjunctive treatment after surgical removal of the MCT. The differences in outcome between dogs with low and high beclin-1 staining MCTs who had adjunctive treatment was analyzed. Survival curves for MST and DFI were constructed for dogs who had one or a combination of vinblastine, radiation (curative or palliative), or toceranib following surgical removal of the MCT. Outcome data from primary MCTs, in addition to primary MCTs together with recurrences (referred to as “All”) were analyzed (Figure 4.3). Strikingly, there is a significant difference in MCT-disease related survival between low and high beclin-1 staining in these treated dogs (Figure 4.3A,B) with primary MCTs alone (p=0.022) and primary combined with recurrent (p=0.005). A Cox proportional hazard analysis calculates a hazard ratio of 7.9 (p=0.053) for dogs with high compared to low beclin-1 expressing
primary MCTs, and a hazard ratio of 10.5 (p=0.024) for primary together with recurrent. The difference in the DFI curves is not significant in either the primary MCTs (p=0.15), or primary together with recurrent (p=0.07). Therefore, although beclin-1 staining showed no prognostic value in MCTs, these results show that the level of beclin-1 staining is able to predict response of MCTs to adjunctive treatment.

Figure 4.3 Kaplan-Meier survival curves for non-AT treated cutaneous MCTs stratified by beclin-1. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05

As subcutaneous MCTs tend to have a better prognosis than dermal MCTs (Thompson et al., 2011a), the predictive value of beclin-1 staining in MCTs separated into dermal and subcutaneous only was examined. The number of primary and recurrent treated dermal tumours is relatively low. However, although the difference is not significant, the dogs with adjunctive
therapy treated dermal tumours had decreased survival in dogs with high beclin-1 staining primary and recurrent MCTs (Figure 4.4)(p=0.092). Similar results are observed with subcutaneous MCTs (Figure 4.5)(p=0.051).

Figure 4.4 Kaplan-Meier survival curves for AT treated dermal MCTs stratified by beclin-1. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.
Figure 4.5 Kaplan-Meier survival curves for AT treated subcutaneous MCTs stratified by beclin-1. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

Given that the number of treated tumours in our analysis is relatively low, it is possible that confounding factors were affecting our results; for example, if high beclin-1 staining MCTs coincidentally were also high-grade. To investigate this further, treated low-grade and treated high-grade cutaneous MCTs were analyzed separately. There were no significant differences in MST or DFI in the low-grade subset of MCTs (Figure 4.6). In high-grade MCTs, there is a clear difference in survival outcome in primary MCTs alone (p=0.011) and combined with recurrent MCTs (p=0.006) (Figure 4.7). Hazard ratios could not be calculated with these data as there were no MCT-related deaths in the low beclin-1 staining group. There was also a significant difference
in the DFI curve in primary and recurrent MCTs (p=0.045).

Figure 4.6 Kaplan-Meier survival curves for AT treated low-grade cutaneous MCTs stratified by beclin-1. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.
Survival was next examined in dermal MCTs categorized according to the Patnaik grading scheme. The low numbers of treated grade I and grade III dermal MCTs precluded a survival analysis for these categories. However, clinically, the grade II MCTs are the group whose prognosis is most difficult to predict, and therefore may also complicate treatment decisions. If a predictive response to adjunctive therapy in grade II tumours is observed, this is likely the most clinically relevant information. The number of primary tumours was quite low, and therefore the analysis was done on primary combined with recurrent tumours (Figure 4.8). High beclin-1 expressing MCTs showed a non-significant decreased MCT-related survival time (p=0.14).

Figure 4.7 Kaplan-Meier survival curves for AT treated high-grade cutaneous MCTs stratified by beclin-1. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05
Figure 4.8 Kaplan-Meier survival curves for AT treated grade II dermal MCTs stratified by beclin-1. (A) shows survival time and (B) shows disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous.

As previously mentioned, the only prognostic indicator determined specifically for subcutaneous MCTs is mitotic count. Therefore, MST and DFI in subcutaneous MCTs with low and high mitotic count was also examined. There were no recurrent low mitotic count subcutaneous MCTs in our dataset, and therefore only primary low mitotic count subcutaneous MCTs were analyzed (7 low beclin-1, and 4 high-beclin-1); there were no significant differences in survival or DFI curves (MST p=0.74, DFI p=0.63; data not shown). The number of adjunctive treated high mitotic count subcutaneous MCTs was too low to conduct an analysis.

Subcutaneous together with dermal MCTs were also analyzed by low and high mitotic count. Similar to the results with the Kiupel grading, in the MCTs with a better prognosis, the low mitotic count MCTs, there were no significant differences in survival or DFI curves (Figure 4.9). However, in the high mitotic count MCTs, there were significant differences in the survival curves in the primary (p=0.037) and primary combined with recurrent (p=0.022) MCTs (Figure 4.10).
Figure 4.9 Kaplan-Meier survival curves for AT treated low MC cutaneous MCTs stratified by beclin-1. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MC, mitotic count; MCT, mast cell tumour; SC, subcutaneous.
In our analyses of the adjunctive treated group of MCTs, all adjunctive treated dogs were included, regardless of the treatment modality. However, there were seven different possible combinations of treatments, and the cases fell into six of these groups (Table 4.1).
In an effort to differentiate which one(s) of these adjunctive treatment responses is being predicted, analyses were first done with groups that had each of the three treatments (with or without other treatments) (Figure 4.11). There was a significant difference in survival curves in the chemotherapy treated dogs (p=0.016), and although not significantly different, the high beclin-1 staining MCTs in the toceranib group appeared to have decreased survival compared to the low beclin-1 staining (p=0.075).
Figure 4.11 Kaplan-Meier survival curves for differently AT treated cutaneous MCTs stratified by beclin-1. (A,C,E) show survival time and (B,D,F) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05

The subgroups of those dogs treated with one of the three adjunctive treatments without any other adjunctive treatment were then compared (Figure 4.12). No significant differences were observed
in the survival curves, although the high beclin-1 expressing MCTs appeared to have decreased survival compared to low beclin-1 expressing MCTs with chemotherapy only and toceranib only treatments, and there was a significant reduction in DFI (p=0.013) in high beclin-1 expressing MCTs treated with chemotherapy (Figure 4.12B).
Figure 4.12 Kaplan-Meier survival curves for single AT treatment cutaneous MCTs stratified by beclin-1. (A,C,E) show survival time and (B,D,F) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05
Survival curves of mast cell tumours given adjunctive therapy, stratified by known prognostic markers

A predictive protein biomarker has not yet been identified for MCTs. As mentioned previously, there is some predictive value in knowing the KIT mutation status. However, given that even MCTs with no identified KIT mutation often have some response to treatment with toceranib, the KIT mutational status is not particularly useful from a clinical point of view, and a predictive protein biomarker could be quite valuable in directing treatment. Our data suggest that beclin-1 staining levels do have predictive value, especially in those MCTs with poorer prognoses (e.g. high-grade or high mitotic count). However, it is also possible that the known prognostic markers have equal predictive value. To investigate this, survival functions in adjunctive treated dermal MCTs stratified by Patnaik grade (data not shown), and adjunctive treated cutaneous (dermal and subcutaneous) MCTs stratified by Kiupel grade, by mitotic count, and by KIT staining pattern was examined. Although KIT staining levels showed no prognostic information, the adjunctive treated cutaneous MCTs were also analyzed after stratification for KIT staining levels. There were no significant differences in survival curves in primary (p=0.16) and primary and recurrent dermal MCTs (p=0.076) stratified by Patnaik grade (data not shown). There were significant differences in survival curves stratified by Kiupel grade (primary, p=0.005; primary and recurrent, p<0.001) (Figure 4.13), mitotic count (primary, p<0.001; primary and recurrent, p<0.001) (Figure 4.14), and KIT pattern (primary and recurrent, p=0.039) (Figure 4.15). There were no significant differences in the survival curves in primary (p=0.94) and primary and recurrent dermal MCTs (p=0.89) stratified by KIT staining levels (data not shown).
Figure 4.13 Kaplan-Meier survival curves for AT treated cutaneous MCTs stratified by Kiupel grade. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MCT, mast cell tumour; SC, subcutaneous. *p<0.05
Figure 4.14 Kaplan-Meier survival curves for AT treated cutaneous MCTs stratified by MC. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; MC, mitotic count; MCT, mast cell tumour; SC, subcutaneous. *p<0.05
Figure 4.15 Kaplan-Meier survival curves for AT treated cutaneous MCTs stratified by KIT pattern. (A,C) show survival time and (B,D) show disease-free interval. The vertical tick-marks correspond to censored data. Survival functions were compared using the logrank test. AT, adjunctive therapy; KP, KIT pattern; MCT, mast cell tumour; SC, subcutaneous. *global p<0.05

**Beclin-1 staining levels predicts response to treatment in canine cutaneous mast cell tumours**

Along with beclin-1 staining levels, this study found that three other factors (Kiupel grade, mitotic count, KIT staining pattern) appeared to have predictive ability, showing significantly different survival curves for combined primary and recurrent adjunctive treated cutaneous MCTs. However, this apparent predictive ability may be confounded by the fact that these are well-recognized prognostic factors, and that MCT behaviour can vary widely from benign to aggressive. In other words, some of the adjunctive treated low-grade MCTs that appear to have a good response to adjunctive treatment (hence attributing predictive ability to the Kiupel grading scheme), may in fact never have progressed regardless of whether treatment was given or not.
Therefore, all the variables that showed apparent predictive ability in a univariable analysis were included in a multivariable analysis to determine which variable was most significantly predictive of response to treatment (Table 4.2). Notably, in primary only adjunctive treated cutaneous MCTs, only beclin-1 staining levels approach significance (p=0.055), and in primary combined with recurrent adjunctive treated cutaneous MCTs, only beclin-1 staining levels show significant predictive ability (p=0.035).

Table 4.2 Multivariable analysis for predictive factors for adjunctive treatment of combined primary and recurrent treated cutaneous (dermal and subcutaneous) MCTs.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Univariable</th>
<th>Multivariable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% confidence interval</td>
<td>p-value</td>
</tr>
<tr>
<td>High beclin-1 (vs. low)</td>
<td>7.92</td>
<td>0.97-64.58</td>
</tr>
<tr>
<td>MC &gt;4 (vs. MC &lt;=4)</td>
<td>7.87</td>
<td>2.02-30.65</td>
</tr>
<tr>
<td>High Kiupel grade (vs. low)</td>
<td>5.30</td>
<td>1.43-19.67</td>
</tr>
<tr>
<td>KIT pattern III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(vs. pattern I)</td>
<td>4.76</td>
<td>1.05-21.46</td>
</tr>
<tr>
<td>(vs. pattern II)</td>
<td>2.89</td>
<td>0.52-15.9</td>
</tr>
<tr>
<td>High beclin-1 (vs. low)</td>
<td>10.53</td>
<td>1.35-81.95</td>
</tr>
<tr>
<td>MC &gt;4 (vs. MC &lt;=4)</td>
<td>7.11</td>
<td>2.25-22.47</td>
</tr>
<tr>
<td>High Kiupel grade (vs. low)</td>
<td>6.70</td>
<td>1.93-23.31</td>
</tr>
<tr>
<td>KIT pattern III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(vs. pattern I)</td>
<td>4.09</td>
<td>1.19-14.06</td>
</tr>
<tr>
<td>(vs. pattern II)</td>
<td>3.08</td>
<td>0.63-14.96</td>
</tr>
</tbody>
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* p-value <0.05

**Discussion**

In this study, beclin-1 was identified as a promising novel predictive biomarker in adjunctive treated canine cutaneous MCTs. Interestingly, although previously identified prognostic markers, such as mitotic count, KIT staining pattern, and Kiupel grade appeared to predict response to therapy in treated MCTs, in a multivariable analysis of these three factors as well as beclin-1 staining, only beclin-1 staining maintained statistical significance. This suggests that beclin-1 staining level is the best independent predictive biomarker. This conclusion is also supported by the fact that within the subgroup of Kiupel high-grade MCTs and the subgroup of high mitotic count cutaneous MCTs, beclin-1 maintains its predictive value.
In contrast to the results from the subgroups with poor prognosis (e.g. Kiupel high-grade and high MC), our analysis shows that within the subgroups with good prognosis, such as Kiupel low-grade MCTs, and low mitotic count MCTs, beclin-1 staining levels are no longer predictive. Beclin-1 staining levels were not prognostic (see Chapter 2). Therefore, it is likely that we will only see a significant difference in outcome in MCTs by beclin-1 staining levels in treated MCTs that have aggressive behaviour and will recur and/or metastasize. That is, in a group of treated MCTs that includes tumours that would not have progressed even without adjunctive treatment, the ability of beclin-1 staining to predict response to adjunctive treatment is significantly diminished.

To date, the only biofactor that has been examined as being potentially predictive, is the c-KIT mutational status and its predictive effect is in response to receptor tyrosine kinase (RTK) inhibitor therapy. In one study, there was a response rate of 69% to toceranib in dogs with recurrent, Patnaik grade II or III MCTs with c-KIT mutations, however, in those without mutations, there was a 37% response rate (London et al., 2009). Another study showed little difference in tumour response to masitinib between those MCTs with and without c-KIT mutations (Hahn et al., 2010), while a recent study found similar results with toceranib treatment (Weishaar et al., 2018). It has been hypothesized that these RTK inhibitors may also be affecting not just unregulated KIT activity, but also activity of other RTKs such as VEGFR2 and/or PDFGRα/β in neoplastic and/or associated stromal cells that may be playing a role in tumour progression. As such, the mutational status of c-KIT has not been widely adopted as a clinically useful predictive marker, and if known, does not typically influence the decision to pursue RTK inhibitor therapy.

This study has identified a predictive marker that may prove useful in deciding whether to pursue adjunctive treatment after surgical removal of a cutaneous MCT, especially for high-grade tumours. Predictive markers play a key role in personalized medicine, which is becoming
increasingly important as the number of different medicines and treatment modalities continues to grow at a high rate. Veterinary clinicians and owners may have a relatively easy time deciding whether to pursue additional treatment for those MCTs with excellent (e.g. 5 mm in diameter, completely excised with wide margins, grade I dermal MCT) or very poor prognoses (e.g. 6 cm in diameter, incompletely excised, grade III dermal MCT). However, the vast majority of dermal MCTs are Patnaik grade II MCTs whose behaviour is difficult to predict. In addition, there are many subcutaneous MCTs whose mitotic count is very close to the cutoff of 4, therefore complicating the decision on whether to pursue additional treatment. Along with other factors that will always play a role in the decision, such as surgical margins, MCT location, owner finances, comorbidities, and anticipated side-effects, knowing the beclin-1 status and therefore whether the MCT has a good or poor chance of responding to treatment may help guide many clinicians and owners in making a choice that is right for them and their dog.

Beclin-1 plays a key role in autophagy, a process that is increasingly being recognized as being dysregulated in neoplasia. Although the upregulation of autophagy is thought to protect some cancer cells and promote cancer growth in times of metabolic, hypoxic, or cytotoxic stress, the role of autophagy in cancer is not straightforward, and likely dependent on the particular microenvironment, stage of development, and neoplastic cell of origin. Monoallelic deletion of the BECN1 gene has also been found in several human cancers, and its loss is actually thought to contribute to tumourigenesis (Gao et al., 1995; Russell et al., 1990; Saito et al., 1993). In human gastrointestinal stromal tumour cell lines, a mutant KIT-driven cancer, knockdown of BECN1 leads to accumulation of mutant KIT (Hsueh et al., 2013). In our study, beclin-1 appears to be playing a protective role and allowing neoplastic cells to survive in the face of treatment.

Beclin-1 staining level has been investigated as a prognostic and predictive marker in multiple human cancers. The role of beclin-1 as a prognostic marker appears to be dependent on
the type of cancer. One meta-analysis found that high beclin-1 staining level indicates a more favourable prognosis in gastric cancer and lymphoma, whereas it had no prognostic value in colorectal, breast, and lung cancers (He et al., 2014). In oral cancers, low beclin-1 staining was associated with a better prognosis (Liu et al., 2015), whereas in high-grade gliomas (Pirtoli et al., 2009) and cholangiocellular carcinomas (Dong et al., 2011), the inverse was true. Two interesting studies in the investigation of beclin-1 staining as a predictive marker found similar results to our study. One study of human colorectal cancer found that high (versus low) beclin-1 staining in neoplastic rectal carcinoma cells was significantly associated with tumour downstaging following neoadjuvant chemoradiation treatment (Zaanan et al., 2015). Another study in esophageal squamous cell cancer showed that patients with tumours that were negative for staining of beclin-1 and another autophagy marker microtubule-associated protein light chain 3 (LC3), had better overall survival, and that LC3 staining level was an independent predictive factor in patients receiving definitive chemoradiation (Chen et al., 2013).

The role of autophagy in canine tumours has not been studied extensively and is not well understood. In one study of canine osteosarcoma cell lines, treatment with a heat shock protein 90 inhibitor induced autophagy (Massimini et al., 2017). Another study examining the protein P62/sequestosome-1 has suggested that autophagy may be important in MCT biology. This protein is a “hub” protein that acts as an adaptor molecule to influence the uptake by autophagosomes of cargo targeted for autophagic degradation. This study showed P62 nuclear and cytoplasmic immunoreactivity was significantly associated with Kiupel low- and high-grade tumours, respectively (Rich et al., 2015). Although no outcome data was available for this study, these results raised the possibility that the cytosolic P62 may be an indicator of increased autophagy in high-grade MCTs. In malignant canine mammary tumours, cytoplasmic beclin-1 staining was reported to be lower in neoplastic cells than in surrounding non-neoplastic mammary epithelial
cells, and low beclin-1 staining was associated with higher histologic grade and increased mitotic activity. Furthermore, decreased cytoplasmic beclin-1 staining was significantly associated with poorer overall survival (Liu et al., 2013). In this present study, in contrast to the results in canine mammary carcinoma, beclin-1 staining level was a purely predictive factor and had no prognostic value.

Although beclin-1 proved to be a statistically significant predictive maker for response to adjunctive treatment (regardless of modality) in our dataset, the limited numbers of treated dogs in each of the seven different categories (each type of treatment alone, and in different combinations) hindered our ability to tease out exactly to which treatment(s) the responses were being predicted. It is certainly plausible that beclin-1 can be cytoprotective for all three modalities employed in the adjunctive treatment of canine MCTs. Conventional cytotoxic drugs are now well known to induce autophagy (Yang et al., 2011), but autophagy has also been shown to help protect cancer cells against radiation therapy (Paglin et al., 2001), as well as treatment with RTK inhibitors (Han et al., 2011). Clinical trials are currently underway to investigate the effect of autophagy inhibitors, such as chloroquine and hydroxychloroquine, alone or in combination with chemotherapies in various human cancers (Ozpolat and Benbrook, 2015; Yang et al., 2011). Phase I clinical trials for combined hydroxychloroquine and doxorubicin in dogs with lymphoma have also been completed (Barnard et al., 2014). Future studies will hopefully show that canine MCTs with high beclin-1 expression may yet respond to current therapies if delivered in combination with autophagy inhibitors.

The limited numbers of adjunctive treated dogs with tissue spots that met the inclusion criteria to derive an H-score for beclin-1 is a limitation in this study. However, given the fact that the results remained statistically significant in a multivariable analysis, in combination with the fact that the results are reasonable in a biological sense, these data are extremely promising.
Another limitation of this study is the retrospective design, and inclusion of the MCTs in the TMA was based on what was available. On one hand, a combination of non-adjunctive treated and adjunctive treated samples from primary and tertiary clinics resulted in lower overall numbers for each studied subgroup; on the other hand, this combination allowed us to investigate the role of beclin-1 as both a predictive and prognostic marker. Although only cutaneous MCTs removed with excisional biopsies were included in the analysis, incompletely excised MCTs were not excluded. Exclusion of incompletely excised MCTs would eliminate all the radiation treated MCTs from our analysis. Additionally, many MCTs do not recur even when incompletely excised, especially those MCTs that are expected to have better prognoses (Smith et al., 2017; Thompson et al., 2011a; Vincenti and Findji, 2017).

Although many of the tumours included in the TMA were removed at a tertiary clinic, much of the follow-up data was obtained from the primary care referral veterinary clinics. A postmortem examination was not always performed, and the presence of local recurrence and metastasis was, in a small number of cases, based on physical rather than histological examination. Future investigation of the role of autophagy as a prognostic marker in canine MCTs should include a prospective analysis with higher numbers of adjunctive treated dogs. Additionally, the staining levels of LC3B, a common autophagy-related marker that has been used to monitor autophagy (Klionsky et al., 2012) could be investigated to lend further weight to the idea that increased autophagy results in poor response to treatment.

In conclusion, this study found that the level of beclin-1 staining in canine cutaneous MCTs was significantly associated with response to adjunctive treatment, particularly in the set of tumours with negative prognostic indicators. This study will hopefully prove useful in not only directing individualized treatment plans in dogs with MCTs, but also in helping to develop
effective treatments for those dogs with non-responsive tumours by combining current therapies with autophagy inhibitors.
Chapter 5: General Discussion

Canine MCTs represent up to 21% of all canine cutaneous cancer (Bostock, 1986; Cohen et al., 1974; Graf et al., 2018; London and Seguin, 2003; Welle et al., 2008). Cutaneous MCTs typically occur as a solitary mass, either in the dermis (dermal MCTs) or in the subcutaneous tissue (subcutaneous MCTs). One extremely important feature of canine MCT biology is that the behaviour of MCTs can range from completely benign to fatal, with aggressive local recurrences and/or widespread metastases within multiple internal organs. Therefore, after surgical excision and histopathologic confirmation of an MCT, many different factors come into play in order for the clinician to recommend, and for the owner to decide, whether adjunctive treatment should be pursued. The more accurate the prognosis for the MCT, the better equipped we are to avoid unnecessary treatments, and negative side-effects in those dogs whose MCT were not going to progress. Additionally, the choice to pursue adjunctive treatments to prevent disease progression in those dogs with aggressive MCTs is made clearer.

Many different factors have been shown to correlate with cutaneous MCT prognosis and clinical outcome (see Chapter 1, Table 1.3). However, the practical factors that are considered the most reliable and clinically useful for prognostication and in directing treatment options, are the Patnaik (grades I, II, and III) and Kiupel (low-grade and high-grade) grading schemes (Kiupel et al., 2011; Patnaik et al., 1984). Although the Patnaik grading scheme only included dermal (and not subcutaneous) MCTs, pathologists unaware of this difference, could give a subcutaneous MCT a poor prognosis using this Patnaik grading scheme, based on perceived ‘invasion’ into the subcutaneous tissue. In recent years, the awareness of these two subsets of MCTs has grown, and efforts are being made, to use mitotic count (as per (Thompson et al., 2011a)) as a prognostic indicator for subcutaneous MCTs.
Although widely used, there are still limitations with both the Patnaik and Kiupel grading schemes. The Patnaik grading scheme is not precisely defined, which can lead to large interobserver variation in grading (Kiupel et al., 2011; Northrup et al., 2005b). The prognostic information for a grade II tumour is also limited; and unfortunately, a large percentage of MCTs are classified as grade II (Patnaik et al., 1984; Sabattini et al., 2015). The Kiupel grading scheme (which included both dermal and subcutaneous MCT in its development) showed improvement over the Patnaik scheme in terms of interobserver variation, however, up to 15% of tumours classified as low-grade by this scheme will go on to recur and/or metastasize (Kiupel et al., 2011; Stefanello et al., 2015). Additionally, the inclusion of subcutaneous MCTs likely hinders the accuracy of the grading scheme, given that subcutaneous MCTs tend to have a better prognosis than dermal MCTs (Sabattini et al., 2015; Thompson et al., 2011a).

The limitations of the Kiupel grading scheme have clearly been recognized, as since its publication, there have been continued efforts to identify novel prognostic biomarkers for canine MCTs. One study examining multiple known prognostic indicators, such as clinical stage, history of tumour recurrence, Patnaik and Kiupel grades, organization of neoplastic cells, mitotic count, Ki-67 index, KIT IHC staining pattern, and c-KIT mutational status, found that only clinical stage and history of tumour recurrence were independent predictors of overall survival (Horta et al., 2018). While it may be true that evidence of recurrence and/or metastases are better predictors of outcome compared to a simple grading scheme alone, the clinical utility of these findings is questionable. The decision to recommend adjunctive treatment in those cases that present with recurrence and/or metastasis is likely straightforward; what is needed, is an accurate prediction of MCT behaviour prior to recurrence and/or metastasis.

My study has overcome a common limitation by gathering clinical outcome data from the dogs whose MCTs I am investigating, enabling us to identify novel and improved prognostic
factors compared to the ones in use. Several publications in the last couple of years have attempted to identify novel prognostic factors for canine MCTs. Although these studies all contribute to our knowledge of MCTs, they all share a significant limitation: they have all attempted to identify novel prognostic factors by making correlations with known prognostic factors, such as mitotic count and histologic grade. This limitation hinders their ability to identify a factor with improved prognostication compared to the ones currently in use. One of the studies includes a genomic profiling investigation of genome-wide DNA copy numbers, that found that DNA copy number aberrations were positively correlated with KIT mutations and grade III Patnaik, and high-grade Kiupel MCTs (Mochizuki et al., 2017). Another study looking at fibroblast activating protein (FAP), expressed by tumour associated fibroblasts, found that high stromal FAP expression also correlated with Patnaik and Kiupel grades, as well as Ki67 index and mitotic count (Giuliano et al., 2017). Another recent study showed that increased glucose uptake (as measured by positron emission tomography/computed tomography) correlated with MCT grade (Griffin et al., 2018). However, the expression of NANOG, a pluripotency factor, in canine neoplastic mast cells, showed no correlation with histological grade, mitotic count, Ki67 index, or KIT pattern (Joselevitch et al., 2018). Yet another recent study examining expression of heat shock proteins (Hsp) found that expression of Hsp32 and Hsp90 did not significantly correlate with KIT staining pattern, histologic grade, or mitotic count (Romanucci et al., 2017).

Mast cell tumour tissue microarray and image analysis

The canine MCT TMA constructed and analyzed in this study has many advantages over previous studies. The TMA included a large number of canine MCTs, and to date, is the largest MCT TMA constructed (with the only other published MCT TMA composed of 42 MCTs (Webster et al., 2006)). It also included both MCTs of dogs with and without adjunctive therapy enabling us to ask whether biomarker staining holds both prognostic and/or predictive ability.
Perhaps most importantly, clinical outcome for many of the MCTs was known, which enabled us to immediately compare whether our newly identified biomarkers have improved prognostic information compared to known prognostic factors, such as mitotic count, KIT IHC staining pattern, and histologic grade.

The use of the high-throughput TMA method allows for simultaneous IHC analysis on large numbers of samples, thereby subjecting the samples to identical experimental conditions and decreasing the cost and time for experiments. There are no set standards for the number of cores to be taken for a TMA, and for the single published MCT TMA, it appears that a single 1 mm core was used for each MCT (area of 0.79 mm$^2$) (Webster et al., 2006). For this study, I included three 0.6 mm cores for each MCT (total area of 0.85 mm$^2$), unless the size of the MCT was a limiting factor, and therefore to avoid coring the entire tumour, only one or two 0.6 mm cores were taken. For three of the biomarkers analyzed in this study by IHC (mast cell tryptase, c-CBL, beclin-1), there was little variability between the cells within a tumour in the staining intensity of the proteins (as shown in Figure 2.8). Therefore, three cores are likely sufficient to analyze canine MCTs, at least for the proteins analyzed here. Future studies examining protein staining that is strongly positive in only a few cells within the tissue spot (for example, Ki-67 or stromal cell immunopositivity) will likely require larger minimum tissue areas and/or different optimization of the digital analysis parameters.

One unforeseen confounding factor in the digital analysis was the tendency of the software to identify non-cellular areas (such as collagen) as neoplastic cells, resulting in extra numbers of falsely identified cells that would be measured as having negative immunopositivity. As some MCTs induce fibroplasia, this was an issue in some of the tissue cores. This limitation was addressed through manual annotation of non-cellular areas to exclude them from the analysis. Excluding large non-cellular areas was relatively straightforward, however, excluding non-cellular
areas in which neoplastic mast cells were widely and evenly dispersed (e.g. cells that are separated by edematous collagen) was more challenging. However, as the proteins evaluated in this study were all relatively homogeneously expressed, and the fact that I divided the MCTs into low versus high staining groups, the chance of having a small number of falsely identified (negative immunostaining) cells influencing the final grouping of low- versus high-expressing groups is low.

This was a retrospective analysis, which meant that certain limitations existed, such as limited choice in which MCTs to include in the TMA, differences in tissue processing between the blocks from Yager-Best versus AHL, variability in our success to acquire follow-up data from the dogs whose MCTs were included, and differences in adjunctive treatments after surgical excision, if pursued at all. In addition, there were low numbers of dogs included within certain subsets that were examined. However, this was a screening analysis to identify potential novel prognostic and/or predictive markers, and, like all high throughput analyses, promising biomarkers will need further confirmation by targeted, independent experiments.

**Comparison of known prognostic markers for canine mast cell tumours**

I initially evaluated grading and mitotic count in the full sections of the tumours and compared survival and disease-free times by these known prognostic factors. Overall, our results fit very well with the previously published reports. The MST and DFI outcome of those dogs with untreated dermal MCTs showed an overall significant difference when stratified by the three Patnaik grades, the set of untreated cutaneous MCTs showed significant differences between low- and high-grade Kiupel MCTs, and the non-adjunctive treated subcutaneous MCTs showed significant differences between low (less than or equal to 4) and high (greater than 4) mitotic count. However, it is important to reiterate that some of subcutaneous MCTs included in the TMA were also included in the Thompson et al. study. One slight difference in our data compared to published reports (Kiupel et al., 2011; Thompson et al., 2011a), is that our group of dogs,
especially low-grade cutaneous and low mitotic count subcutaneous MCTs tended to have higher rates of MCT-related death or disease compared to those groups of dogs (i.e. low-grade and low mitotic count) in the published literature. This could be explained by the fact that many of the tumours in our TMA were sourced from a tertiary clinic, which may have resulted in a bias toward more aggressively acting tumours clinically, despite a histopathological diagnosis of low-grade or low-mitotic count, or ones that were in an area that were difficult to resect and were thus referred to board-certified surgeons.

Although the overall survival functions were significantly different between KIT IHC staining patterns I, II, and III, unlike previously published reports (Kiupel et al., 2004; Webster et al., 2007), I found no significant difference between patterns I and II. One possible reason was that although in many tumours the neoplastic mast cells all showed the same staining pattern, some of tumours had a mixture of two, or even all three staining patterns. Since there are as yet no published guidelines on how to evaluate tumours with a mixture of patterns (e.g. choose the predominant pattern versus choose the pattern with the poorest prognosis), it is possible that differences in evaluating the staining pattern may have led to differences in stratification by outcome.

**New prognostic and predictive factors for canine cutaneous mast cell tumours**

Our next step in the analysis of the TMA was to identify novel prognostic markers for canine MCTs. I found that tryptase staining levels was prognostic in primary, untreated, low-grade cutaneous MCTs and low mitotic count subcutaneous MCTs. In both cases, lower tryptase levels were significantly associated with decreased time to recurrence and/or metastasis. In the case of c-CBL, I found a trend toward high c-CBL expressing MCTs having decreased MST in adjunctive treated subcutaneous MCTs. It is interesting to note that in both these cases, there were differences in either MST (c-CBL) or in DFI (tryptase), but not both MST and DFI for either factor.
One would typically expect recurrence and metastasis to follow along with MCT-related survival, resulting in similar results from both these analyses. Perhaps in the case of tryptase, the recurrences or metastases that occurred in the low-tryptase group of MCTs were not so aggressive as to cause the death of the dog. Alternatively, it is possible that these recurrences or metastases occurred in older dogs, or those with co-morbidities, and were thus euthanized or died due to non-MCT related disease. In the case of decreased MST (but not decreased DFI) in high c-CBL expressing adjunctive treated subcutaneous MCTs, perhaps this discrepancy can be explained by the fact that there were dogs with MCTs (both low and high c-CBL expressing ones) that had MCT-related disease at or soon after surgical removal (as evidenced by the sharp drop at the beginning of the curves). Future analyses including more dogs with MCTs that do not already have MCT-related disease at or soon after surgical removal, may show a difference in DFI based on c-CBL staining intensity.

The staining of c-CBL appears to be predictive of response to treatment in subcutaneous, but not dermal, MCTs. Although many previous studies have grouped them together, the fact that two distinct groups of cutaneous MCTs exist is becoming more appreciated in recent years. It has been shown that subcutaneous MCTs are generally less aggressive than their dermal counterparts, and it was hypothesized that this may relate to the influence of the surrounding adipose tissue (Thompson et al., 2011a). Adipose tissue does not merely provide lipid storage but is an active endocrine organ that produces metabolites and bioactive peptides (so-called ‘adipokines’) that play important roles in health and disease (Fasshauer and Bluher, 2015). Another possibility to explain the difference in outcome, is perhaps that subcutaneous MCTs actually develop from mast cell precursors arising from adipose tissue, as has been shown to occur in mice (Poglio et al., 2010), rather than from bone marrow. It is also possible that the different predictive ability of c-CBL
between dermal and subcutaneous MCTs relates to the differences in development and the microenvironment of the mast cells between these two sites.

The staining level of beclin-1 was found to not have prognostic ability but did show very promising predictive ability in both dermal and subcutaneous treated MCTs, particularly in the subsets of tumours with poorer prognoses (e.g. high-grade or high mitotic count). Although there was no significant correlation between c-CBL and beclin-1 H scores in our cutaneous MCTs (data not shown), it is perhaps not surprising that both high c-CBL and high beclin-1 levels predict poor response to treatment. Both beclin-1 and c-CBL function in protein degradation pathways. Beclin-1 is a key player in the autophagy-lysosome pathway, while c-CBL is a ubiquitin protein ligase that ubiquitinates and targets many proteins for degradation via the ubiquitin proteasome system.

There is also overlap between the autophagy-lysosome and the ubiquitin proteasome pathways. Not only does c-CBL act as an E3 ubiquitin ligase, it also functions as an adaptor protein (independent of its ligase function) and regulates diverse signaling pathways, including the focal adhesion kinase (FAK)-Src pathway which mediates signal transduction by integrins, a family of cell surface receptors (Kuang et al., 2013). One way that neoplastic cells exploit autophagy to enhance survival when the FAK-Src pathway is perturbed, is through the autophagic destruction of active Src, a tyrosine kinase. c-CBL acts as a cargo receptor for Src (together with the autophagosome protein LC3B) to help recruit Src to autophagosomes (Sandilands et al., 2012). Interestingly, depletion of c-CBL, which blocked Src recruitment to autophagosomes, resulted in cell death in a squamous carcinoma cell line deficient in FAK. This recruitment function of c-CBL was found to be independent of its E3 ligase domain. At the time of publication of the study by Sandilands et al., it was hypothesized that c-CBL may act as a cargo receptor for other autophagy substrates, and perhaps its ubiquitin ligase activity may also be involved in autophagic destruction of other proteins (Cecconi, 2011). Indeed, c-CBL was recently found to act as a cargo receptor to
recruit another protein, paxillin (a cell scaffold and cell signaling protein that can form a complex to FAK), to autophagosomes (Chang et al., 2017). This autophagic targeting plays an important role in regulating cell-matrix adhesion and cell locomotion in human breast cancer cells (Chang et al., 2017). Taken together, these results suggest that c-CBL may be a potential target in future therapies for both human and canine cancers.

Arguably the most clinically relevant discovery from this analysis is that high beclin-1 levels are predictive of poor response to treatment. This is because clinical trials are already underway to investigate whether administration of autophagy inhibitors, such as chloroquine and hydroxychloroquine, can improve outcome in various human cancers (Ozpolat and Benbrook, 2015; Yang et al., 2011). Moreover, a phase I clinical trial for hydroxychloroquine has been completed in dogs with lymphoma (Barnard et al., 2014). It is tempting to hypothesize that the administration of autophagy inhibitors together with current adjunctive treatments will help improve response to treatment in high-grade (or high mitotic count), high beclin-1 expressing MCTs.

Beclin-1 appears to be a strong predictive factor in canine MCTs, with low beclin-1 expressing MCTs responding well to adjunctive treatment, as evidenced by longer MCT-related survival and disease-free times. However, it would still be interesting to investigate whether treatment can actually induce increased beclin-1 levels in some tumours, leading to resistance to vinblastine or toceranib. Resistance to RTK inhibitors in canine MCTs can occur during long-term treatment, and proposed mechanisms include the development of secondary c-KIT mutations, increased expression of KIT, and upregulation of alternative pro-proliferative pathways (Halsey et al., 2014; Klopfelisch et al., 2012; Kobayashi et al., 2015; London and Thamm, 2013; Nakano et al., 2017). A recent report has found that resistance in a canine MCT cell line to the RTK inhibitor imatinib is related to decreased ubiquitination and subsequent increased half-life of KIT.
The authors concluded that the decreased ubiquitination was not related to downregulation of an ubiquitin ligase such as c-CBL, as a deubiquitinating enzyme inhibitor was enough to inhibit KIT overexpression. They therefore inferred that the decreased ubiquitination was instead associated with activation of a deubiquitinating enzyme. It would be interesting and worthwhile to investigate whether beclin-1 upregulation could be implicated for those MCTs that acquire resistance when treated with RTK inhibitors (London et al., 2009).

**Summary and conclusions**

This TMA was successfully used as a high-throughput screening tool to identify tryptase, a novel prognostic maker for low mitotic count subcutaneous MCTs, and two predictive protein biomarkers in canine MCTs, c-CBL and beclin-1. Based on the promising results shown here, future experiments are warranted. Prospective analyses to investigate the role of tryptase as a prognostic marker in subcutaneous MCTs, and the roles of c-CBL and beclin-1 as predictive markers for response to adjunctive treatment should be conducted to confirm our results. Particularly to confirm predictive markers, the advantage of a prospective analysis is that the treatment protocols can be standardized, reducing the possibility of confounding variables, and hopefully there will also be increased follow-up times. The effect of autophagy inhibitors can also be investigated in MCT cell lines, as well as in clinical trials, in combination with therapy that is currently in use.

An advantage of the TMA that was constructed for this study, is that it can still be used to identify other prognostic and predictive factors. As with conventional tissue blocks, the cores within the TMA will maintain antigenicity, and further sectioning can be done at any time. The fact that both primary, untreated tumours, and recurrent and treated tumours, are included in this TMA is a strong advantage. The number and different types of adjunctive treatments used
clinically is always on the rise, not just for human cancers, but also for canine cancers, and considering that millions of dogs in the world are considered part of the family, there will be increasing numbers of dogs with MCTs seeking treatment. Having predictive factors to help direct treatment, thus advancing personalized medicine in these affected dogs will be of immense value to the dogs, their owners, and clinicians. In addition, although aggressive mast cell neoplasms are rare in humans, the value of comparative medicine between human and canine mast cell cancer is increasingly being recognized (Willmann et al., 2019), and the findings presented here may also one day benefit people afflicted with systemic mastocytosis.

This work is the first to identify two predictive protein biomarkers for canine cutaneous MCTs, beclin-1 and c-CBL, and the first to identify a potential prognostic marker specifically for low-grade MCTs, mast cell tryptase. It is my hope that this study will help to improve the success of therapy and prolong the lives for the large number of dogs who will develop aggressive cutaneous mast cell tumours.
References


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Takeuchi, Y., Fujino, Y., Watanabe, M., Takahashi, M., Nakagawa, T., Takeuchi, A., Bonkobara, M., Kobayashi, T., Ohno, K., Uchida, K., Asano, K., Nishimura, R., Nakayama, H., Sugano, S., Ohashi, Y., and


Appendix

Table A.1 Summary of clinical data of MCTs included in the TMA. The MCTs in the “Other” category include deep/intramuscular, mucocutaneous, penis, and nasal planum.

<table>
<thead>
<tr>
<th></th>
<th>Dermal</th>
<th>Subcutaneous</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of MCTs</strong></td>
<td>110</td>
<td>106</td>
<td>28</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>Mean (range)</td>
<td>7.1 (2.4 - 14.0)</td>
<td>7.9 (2.4 - 13.2)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>Male</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Male neutered</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female spayed</td>
<td>43</td>
<td>48</td>
</tr>
<tr>
<td><strong>Top breeds</strong></td>
<td>Labrador retriever</td>
<td>11</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Mixed breed</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Boxer</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Golden Retriever</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td><strong>Type of sample</strong></td>
<td>Biopsy</td>
<td>110</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>Post-mortem</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Type of tumour</strong></td>
<td>Primary</td>
<td>100</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Recurrence</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

MCT, mast cell tumour

Table A.2 Summary of survival function p-values for untreated MCTs stratified by beclin-1 expression level. The p-values represent the logrank comparison between low and high beclin-1 expressing MCTs for survival time and disease-free interval in different subsets of primary, untreated, tumours.

<table>
<thead>
<tr>
<th>Categories of primary, untreated MCTs</th>
<th>Logrank test p-value</th>
<th>Survival</th>
<th>Disease-free interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-grade cutaneous</td>
<td>0.87</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>High-grade cutaneous</td>
<td>0.60</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>KIT I/II cutaneous</td>
<td>0.82</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>KIT III cutaneous</td>
<td>0.62</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Grade II dermal</td>
<td>0.37</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Grade III dermal</td>
<td>0.43</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Low mitotic count subcutaneous</td>
<td>0.63</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>High mitotic count subcutaneous</td>
<td>0.61</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

MCT, mast cell tumour