PhD THESIS

Canine mast cell tumor. Epidemiology, diagnosis and new therapeutic opportunities

SUMMARY OF THE PhD THESIS

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INTRODUCTION

Mast cells are cells derived from the bone marrow, but which become mature only in the target tissues with mucocutaneous localization, in contact with various factors. They play a role in the immune system and contain many active molecules such as histamine, heparin and cytokines.

The canine mast cell tumor is described as having an unpredictable biological behavior, which causes us to always consider the possibility of increased malignancy. Tumoral modification of mast cells is common in dogs and cats, but is also present in other species, including humans. The main form of manifestation of mast cell tumors in dogs is that of skin or subcutaneous tumors, with visceral or systemic forms also being found.

Canine cutaneous mast cell tumor represents 16-21% of skin tumors in dogs, so it can be considered a common tumor, of great importance in the differential diagnosis of skin masses. The diagnosis of this tumor pathology can be made by clinical examination, cytological examination and histopathological examination. Although the cytological examination can easily observe both the characteristic appearance of mast cells and intracytoplasmic granules, as well as some malignancy criteria, the confirmation of the diagnosis is always made using histopathological examination, using existing classification systems in the literature. By assigning a tumor to a grade and corroborating it with clinical aspects such as superficial ulcerations, localization, the presence of paraneoplastic syndromes and the presence of post-excision surgical safety margins, we can state a prognosis.

Research on prognostic molecular markers for mast cell tumors is extensive. However, many unanswered questions remain regarding the genes whose mutations lead to these tumors. For example, the KIT gene mutation is not detected in the vast majority of cases. Recently, new studies have highlighted the importance of the intracellular signaling pathway called JAK-STAT, demonstrating the role of component proteins in the oncogenesis of many types of tumors in both humans and animals. An upward trend is the detection of molecules that act actively and directly on gene transcription. The aim of these studies is to obtain specific therapy protocols, taking into account the fact that many types of tumors, including mast cell tumors, do not currently benefit from this.

STRUCTURE OF THE THESIS

The paper entitled "Canine mast cell tumor. Epidemiology, diagnostic and new therapeutic opportunities" contains a number of 126 pages being drafted under the current normative and is structured into two parts.

The first part, "The review of the literature", is divided into eight chapters and extends over 31 pages. This part summarizes general aspects regarding the structure and function of mast cells, the pathogenesis and epidemiology of canine mast cell tumors, presenting the clinical aspects in canine cutaneous and subcutaneous mast cell tumors. The main diagnostic techniques are described (cytology, histopathology and immunohistochemistry), and also the molecular markers that have been used up to date, in order to establish a prognosis. The last chapter in this part characterizes the JAK2-STAT3 intracellular signaling pathway and its involvement in oncogenesis.

The second part of the thesis, “Personal contributions”, extends to 62 pages and is divided into six chapters. In this part we present the objectives and purpose of research, materials and methods used, results obtained and discussions, comparing our results with the literature. The results are summarized in the form of 41 figures and 4 tables.

In **Chapter 9** we emphasize on the motivation and main objectives of this study. **Chapter 10**, entitled " Epidemiological study of the canine mast cell tumor" was performed using data obtained from the database of the Department of Anatomic Pathology, Necropsy and Forensic Medicine of the Faculty of Veterinary Medicine, Cluj-Napoca, Romania. The study covers a period of 15 years (2005-2020), having analyzed the incidence of this tumor depending on the breed of dogs, the average age of the dogs when diagnosed, the sex predisposition, the main anatomical locations affected, the average time period of tumor evolution and the average size of the tumors. **Chapter 11**, entitled “The histopathological diagnosis of canine mast cell tumors”, aims to describe the main histological features that define this tumor type and to reclassify all tumors identified in the databases of the Pathology Department at FVM Cluj-Napoca, by using a modern classification system (Kiupel), in order to decrease the subjectivity of the examining pathologist. **Chapter 12** aimed to evaluate the immunohistochemical expression of STAT-3 in canine cutaneous mast cell tumors. A large number of representative skin mast cell tumors, previously diagnosed histopathologically using the Kiupel system, were selected. We have identified the best immunohistochemical technique and antibody dilution, giving the best results, with a high degree of specificity. Subsequently, the evaluation of how tumoral mast cells are labeled using this antibody was performed and an attempt was made to correlate the immunohistochemical labeling with the Kiupel histological grade of the

tumors. **Chapter 13** consists of an in vitro study, which aims to evaluate the inhibitory/anti-tumor effect of a molecule called JSI-124, on a canine mast cell tumor model by using flow-cytometry and confocal microscopy. **Chapter 14** outlines the general conclusions derived from this doctoral research and chapter 15 highlights the aspects of originality, the innovative contributions of the thesis and the recommendations.

RESULTS OF THE RESEARCH

Chapter 10

The epidemiological study was performed in order to analyze the data from the last 15 years (period 2005 - 2020) regarding the canine mast cell tumor within the Faculty of Veterinary Medicine Cluj-Napoca. The aim was to obtain data with statistical relevance and compare it with existing literature. The diagnosis was determined by cytopathological and / or histopathological examination, having identified a number of 161 cases of mast cell tumors. In the present study, 40 dog breeds were identified, with both cutaneous and subcutaneous forms of tumors. The breeds identified are: American Bully, American Staffordshire Terrier, Beagle, Bichon, Bobtail, Boxer, German Shorthaired Pointer, Bullmastiff, American Bulldog, English Bulldog, French Bulldog, Poodle, Chihuahua, Chow-Chow, Central Asia Shepherd, Caucasian Shepherd, Bern Shepherd, German Shepherd, Border Collie, Cocker Spaniel, Dogo Argentino, Great Dane, Fox Terrier, Golden Retriever, Husky, Lagotto Romagnolo, Labrador Retriever, Pekingese, Pitbull, Pug, Hungarian Puli, Rottweiler, Schnauzer, English Setter, Shar-Pei, Dachshund, Hungarian Vizsla, West Highland White Terrier, Yorkshire Terrier and mixed-breed. The highest number of cases was recorded in the Mixed-breed (n = 25), followed by Boxer (n = 17), Labrador Retriever (n = 11), Golden Retriever (n = 10) and Shar-Pei (n = 9). This result can be explained, on one hand, by the uncontrolled breeding of dogs and, on the other hand, by the large population of stray dogs belonging to the mixed-breed in this country. A number of 89 cases belong to other breeds (55%), with a much lower individual incidence.

In this study, canine mast cell tumors were identified in patients aged 9 months to 18 years old (y.o). We identified the highest number of cases (n = 41, 25.4%) belonging to the age group 7-8 y.o. The following age groups, in descending order of the number of cases, are: 5-6 y.o. (36 cases, 22.3%), over 10 y.o. (34 cases, 21.1%), 9-10 y.o. (26 cases, 16.1 %), 3-4 y.o. (13 cases, 8%), 1-2 y.o. (10 cases, 6.2%) and under 1 y.o. (1 case, 0.6%). The average age at which individuals develop mast cell tumors

in this study is 7.7 years old, similar to the data found in the literature. Thus, we can say that dogs over 5 years of age are more affected, without excluding the appearance of tumors at younger ages. The age of the dogs taken into study has a direct influence on the incidence of tumors. The determined correlation coefficient is significantly positive, between age and tumor incidence having established a linear regression; so that the transition to a higher age category influences an increase of almost 3-fold in the number of individuals with tumors. The gender differences are not remarkable, having identified 91 males (56.5%) and 70 females (43.4%). By examining the total number of MCTs (n = 159) of which we obtained data on tumor distribution, we observed that they presented mainly as single masses (n = 141, 88.6%) and less as multicentric tumors (n = 18, 11.3%). Multicentric mast cell tumors were noted in the Pitbull (n = 2, 11.1%), Labrador Retriever (n = 2, 11.1%), Mixed-breed (n = 2, 11.1%), Dachshund (n = 2, 11.1%) and Hungarian Puli, Bullmastiff, Boxer, German Shepherd, American Bulldog, Pug, Dogo Argentino, American Bully, Shar-Pei, Rottweiller with only one case each (5.5%).

The most frequently affected anatomical region was the trunk, a number of 63 unique tumors (53%) showing this location. The localization on the limbs was second in number of identified cases (n = 38, 32%), followed by the head and neck (n = 18, 15%). The data regarding the location of the tumor / tumors was not complete, having identified a number of 39 cases in which the affected anatomical region was not specified, as it was missing from the anamnesis sent by the owners or veterinary clinics. The time period from the onset of the lesion to the establishment of the anatomopathological diagnosis was recorded in a number of 34 cases (21%). Thus, we managed to calculate the average time period recorded from the onset of the lesion until the diagnosis, which is 10.7 months (with limits of 2 weeks - 24 months).

The size of the tumors was recorded in a number of 84 cases, the average diameter of the MCTs being 5.4 cm (with limits of 0.3 - 30 cm).

Chapter 11

In this study, 118 canine mast cell tumors were introduced, respectively tissue samples processed using the paraffin inclusion technique. All blocks of paraffin tissue that were diagnosed as canine mast cell tumors in the period 2005-2020 were identified. Histopathological examination remains the "gold standard" method in stating a definite diagnosis and prognosis in the case of canine mast cell tumors. Although the purpose of this study was to classify cutaneous mast cells (n = 101, 85.5%), we also identified a number of 17 (14.4%) subcutaneous tumors that do not undergo the same classification system. An important feature of the Kiupel system is that the individual presence of any of the following criteria: number of mitoses (at

least 7 mitoses / 10 HPF), number of multinucleated cells (at least 3 cells / 10 HPF, minimum 3 nuclei), the number of bizarre nuclei (at least 3 bizarre nuclei / 10 HPF) and the evidence of karyomegaly (if the diameter of at least 10% of the tumor mast cells is at least twice as large as normal), automatically results in the diagnosis of that MCT as being of high grade. Thus, using this method, we diagnosed a number of 53 low-grade tumors (52.5%) and 48 high-grade tumors (47.5%). Considering the 101 cases of skin mast cell tumors diagnosed histologically, we aimed to establish which breeds were most affected be either high- or low-grade tumors. We observed that out of the total number of 53 low-grade tumors, most tumors were found in the Mixed-Breed (n = 7, 13.2%), Labrador Retriever (n = 6, 11.3%), Boxer and Golden Retriever (n = 5, 9.4% each), respectively Poodle and Pug (n = 4, 7.5% each). Regarding the incidence of high-grade tumors, we noticed that the most affected breeds were the Mixed-Breed (n = 8, 16.6%), Boxer and German Shepherd (n = 4, 8.3% each), respectively Labrador Retriever and Pug with a number of 3 cases each (6.2%).

Chapter 12

This study included 40 cases of canine cutaneous mast cell tumors that were diagnosed in the Department of Anatomic Pathology, Necropsy and Forensic Medicine of the Faculty of Veterinary Medicine, Cluj-Napoca, Romania. These tumors had different locations. Tissue samples were initially processed by indirect technique, using the Leica BondMaxTM automated immunohistochemistry system (Leica Biosystems Melbourne, Bond Max model, M2 12154 series), but the results were not satisfactory, with histological sections showing numerous processing artifacts. We resorted to sample processing by manual technique, and the dilution of the primary antibody that revealed the best results turned out to be 1:50. Evaluation of STAT3 expression was performed using an immunoassay intensity grading system. It was determined to be: 0 - negative, 1 - weak, 2 - moderate, 3 - strong. A total of 10 high power fields (40X objective) were evaluated to determine the degree of cell labeling and to be able to accurately determine the score assigned to each immunohistochemical sample and to eliminate the possibility of processing-induced variability. All canine skin mast cell tumor samples included in this study, as well as the positive control, were immunohistochemically labeled for STAT3. In some situations, we noticed a discrete immunoreactivity in the cell nucleus, but quantifying this aspect proved difficult due to the abundant cytoplasm marked positively, so that, most of the time, the nucleus was masked by it. In a small number of samples, we observed a discrete immunoreactivity of the intra-tumoral vascular endothelium, we explain this endothelial immunoreactivity by accentuated intra-tumoral neo-

angiogenesis, in response to an increased expression of VEGF under the influence of cytokines.

Some immunohistochemical samples showed a positive, intense and diffuse marking of the fibroblast cytoplasm, located especially at the edges of the tumor formation itself or in those areas where tumor-induced collagenolysis was extensive. This is also explained by the effect of cytokines (IL-6 / IL-11) and tumor inflammation on the intrinsic JAK2-STAT3 cell signaling pathway, STAT3 dimerization and phosphorylation, nucleus translocation, and gene transcription initiation. Of the 40 canine skin mast cell tumors included in this study, 26 were diagnosed histopathologically as low grade (Kiupel) and 14 were diagnosed as high grade (Kiupel). Referring to the 26 low-grade tumors, we observed that 5 of them (19.23%) had a score of immunoassay intensity of 1, 16 cases (61.5%) had a score of 2 and 5 cases (19.23%) had the score 3. Regarding tumors diagnosed histopathologically as high grade, 3 of them (21.4%) received an immunoassay score of 1, 2 cases (14.28%) received the score 2 and 9 cases (64.28 %) received the score 3. The results obtained indicate that a large proportion of low-grade mast cell tumors tend to be marked with moderate intensity (score 2), while the vast majority of cases of high-grade cutaneous tumors were strongly marked (score 3). However, from a statistical point of view, no significant positive correlations were observed between the intensity of STAT3 immunolabeling and the histological grade of tumors (r = 0.281).

Chapter 13

The biological material was represented by the 2 stable cell lines of canine mast cell tumor, NI-1 (immature myeloid cells, precursors of mast cells) and C2 (mature cells, derived from a canine skin tumor). The experimental groups were established as follows: one untreated control group from each cell line (NI-1 and C2) and three groups from each cell line with inhibitor in a concentration of 800nM/l, 400nM/l and 200nM/l, respectively. Cells were kept in contact with the inhibitor for 24 hours at 37 ° C, then labeled with the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, Darmstadt, Germany).

The JSI-124 inhibitor (Cucurbitacin I) resulted in a dose-dependent increase in the percentage of cells in early apoptosis (Q1) in the NI-1 cell line. By comparing the untreated control group, where we identified 49.4% of cells in early apoptosis, with the other experimental groups, we observed that a concentration of 200nM/l in the inhibitor led to an increase of this percentage to 52.8% (by 3.4% more than the control), the concentration of 400nM/l raised the percentage to 66.9% (by 17.5% more than the control), and the concentration of 800nM/l brought it to a percentage of 71.5% (by 22.1% more than the control). The percentage of viable cells decreased

visibly, being 45.7% in the control group, 32.6% in group C200, 22.1% in group C400 and 10.4% in group C800. Combining the percentage of cells in early apoptosis with that of cells in late apoptosis, we determined that 65% of the cells in group C200 (12% more than in the control group), 75.9% of the cells in group C400 (22.7% more) more than in the control group) and 86.6% of the cells in group C800 (33.4% more than in the control group) were in apoptosis. Referring to group C200, where the percentage of apoptotic cells was 65.2%, doubling the dose (group C400) led to an increase in the number of apoptotic cells by 16.41%. Regarding group C800, quadrupling the dose of inhibitor led to an increase in the percentage of apoptotic cells by 32.82% compared to group C200.

The JSI-124 inhibitor (Cucurbitacin I) resulted in a dose-dependent increase in the percentage of cells in early apoptosis (Q1) in the C2 cell line. Comparing the untreated control group, where we identified a percentage of 36.9 cells in early apoptosis, with the other experimental groups, we observed that a concentration of 200nM/l of inhibitor increased this percentage to 60.6% (by 23.7% more than the control), the concentration of 400nM/l raised the percentage to 72.4% (by 37.5% more than the control), and the concentration of 800nM/l brought it to a percentage of 77% (by 40.1% more than the control). The percentage of viable cells decreased visibly, being 57.4% in the control group, 35.4% in the C200 group, 24.2% in the C400 group and 19.2% in the case of the C800 group. By combining the percentage of cells in early apoptosis with that of cells in late apoptosis, we determined that 63.5% of the cells in group C200 (21.3% more than in the control group), 75.3% of the cells in group C400 (33.1% more) more than in the control group) and 80.7% of the cells in group C800 (38.5% more than in the control group) were in apoptosis. Referring to group C200, where the percentage of apoptotic cells was 63.5%, doubling the dose (group C400) led to an increase in the number of apoptotic cells by 18.56%. For group C800, quadrupling the dose of inhibitor led to a 27% increase in the percentage of apoptotic cells compared to group C200.

GENERAL CONCLUSIONS

1. Epidemiological results of the study of canine mast cell tumors in the period 2005-2020:

- Within the Department of Pathology, a number of 161 tumors from the canine species were diagnosed;
- Most cases were recorded in the Mixed-Breed (n = 26, 15.5%), Boxer (n = 17, 10.5%), Labrador Retriever (n = 11, 6.8%), Golden Retriever (n = 10, 6.2%) and Shar-Pei (n = 9, 5.5%);
- Patients who developed this type of tumor were aged between 9 months and 18 years old, the highest share of cases (n = 41, 25.4%) belonging to the age group 7-8 years old, between the age of the animals and the incidence of MCT there is a significant positive relationship ($r = 0.8913$; $p < 0.01$);
- Gender differences are not remarkable, having identified a number of 91 males (56.5%) and 70 females (43.4%);
- Mast cell tumors presented mainly as single masses (n = 141, 88.6%) and less as multicentric tumors (n = 18, 11.3%);
- The most frequently affected anatomical region was the torso (n = 63, 52.9%), followed by the limbs (n = 38, 31.9%), head and neck (n = 18, 15.1%);
- The average time period recorded from the onset of the lesion until the diagnosis was established was 10.7 months (with limits of 2 weeks - 24 months);
- The average diameter of the MCT was 5.4 cm (with limits of 0.3 - 30 cm).

2. The number of cutaneous mast cell tumors diagnosed histopathologically was much higher than the number of subcutaneous ones (n = 101, 85.5% versus 17, 14.4%).

3. Classification of tumors using a modern method, proposed by Kiupel et al. in 2011, increases the objectivity of the examining pathologist and facilitates the establishment of a correct and concrete diagnosis by drawing specific characteristics of differentiation between low-grade MCT and high-grade MCT.

4. A large number of low-grade MCTs were diagnosed in the Mixed-Breed (n = 7, 13.2%), Labrador Retriever (n = 6, 11.3%), Boxer and Golden Retriever (n = 5, 9.4% each), respectively Poodle and Pug (n = 4, 7.5% each).

5. High-grade MCT was identified in large numbers in the breeds including the Mixed-Breed (n = 8, 16.6%), Boxer and German Shepherd (n = 4, 8.3% each), respectively Labrador Retriever and Pug with a number of 3 cases each (6.2 %).

6. All canine skin mast cell tumors that were included in the study tested positive for STAT3. Low-grade tumors tend to have a moderate intensity of immunolabeling, and high-grade ones generally showed increased immunoreactivity.

6. There are no significant positive correlation between the intensity of STAT3 immunolabeling and the histological grade of tumors (r = 0.281).

7. Discrete immunoreactivity of the intra-tumoral vascular endothelium and a positive, intense and diffuse labeling of the fibroblast cytoplasm can be observed by using the anti-STAT3 IgG1 monoclonal antibody on samples from the canine species.

8. The JSI-124 inhibitor (Cucurbitacin I) is able to induce *in vitro* the onset of apoptosis in NI-1 and C2 cell lines from leukemic and cutaneous mast cell tumor patients, in all tested doses.

9. The concentration of inhibitor that revealed the best results in inducing apoptosis in mast cells in the NI-1 and C2 cell lines was the dose of 400nM / l.