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EVALUATION OF ACETYLATED HISTONES 3 AND 4 AND HISTONE DEACETYLASES 1, 2 AND 6 IN CUTANEOUS T-CELL LYMPHOMA IN DOGS

Oscar Rodrigo Sierra Matiz

Médico Veterinário

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Oscar Rodrigo Sierra Matiz

Orientadora: Prof. Dra. Mirela Tinucci Costa

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TÍTULO DA TESE: EVALUATION OF ACETYLATED HISTONES 3 AND 4 AND HISTONE DEACETYLASES 1,2 AND 6 IN CUTANEOUS T-CELL LYMPHOMA IN DOGS

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OSCAR RODRIGO SIERRA MATIZ –born in Bogotá, Colombia, on april the 15th of 1987, child of Antonio José Sierra Caviedes and Ruth Jesus Matiz Rodriguez. He studied Veterinary Medicine in La Salle University, Bogotá, Colombia and took his degree in December 2010 under the supervision of Dr. Oscar Javier Benavides. He worked as a resident of internal medicine at the Dover Veterinary Clinic in Bogotá, Colombia from 2011 to 2013. He performed international externships in the oncology department of *Madison-Wisconsin University*, under the supervision of Dr. David Vail in 2013, in the *Coral Springs Animal Hospital*, under supervision of Dr. Francisco Alvarez in 2014 and in *Purdue University*, under supervision of Dr. Michael O. Childress, in 2015. He started his Master of Science (MSc.) studies in 2014 in the Faculty of Agricultural and Veterinary Sciences under the Graduate Program of Veterinary Medicine, area of Veterinary Medical Clinics of the São Paulo State University – UNESP, Campus of Jaboticabal under the supervision of Profa. Dra. Sabryna Gouveia Calazans. In March 2016, he started his Doctoral studies (PhD.) under the same Graduation Program at the same institution under the supervision of Profa. Dra. Mirela Tinucci Costa. During his Postgraduate studies, he was elected as a monitor in the *lato sensu* Posgraduate course of Veterinary Oncology in the Bioethicus Institute, Botucatu, SP from 2014 to 2016 and later, from 2017 to 2018, in the 2nd Course of Oncologic and Reconstructive Veterinary Surgery at the São Paulo State University – UNESP, Campus of Jaboticabal. Since 2014 until now he is attending as a voluntary in the Veterinary Oncology Service (SOV) of the Veterinary Hospital “Governador Laudo Natel” and has published several articles and scientific abstracts in international and national journals and annals of congress, as well as has participated as an author in the oncology section of book chapters including “Oncologia em cães e gatos”, “Medicina Felina Essencial” and “Dia a Dia” (published in Portuguese). Sierra Matiz has been supported by the Coordination for the Improvement of Higher Education Personnel – CAPES Foundation during his Posgraduate studies at the UNESP – Campus of Jaboticabal.

“La vida no es lo que uno vivió, sino la que uno recuerda y cómo la recuerda para contarla”

Gabriel García Márquez

À memória de Antonio e Elkin, e aos meus anjos nesta terra Ruth e Sandra

A dedico

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CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Avaliação da acetilação e da expressão das enzimas HDAC1, HDAC2 e HDAC6 como mecanismo epigenetico em linfoma cutâneo de cães"**, protocolo nº 019049/17, sob a responsabilidade da Prof.ª Dr.ª Sabryna Gouveia Calazans, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 07 de dezembro de 2017.

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AVALIAÇÃO DAS HISTONAS ACETILADAS 3 E 4 E DAS HISTONAS DESACETILASES 1, 2 E 6 EM LINFOMA CUTÂNEO DE CÉLULAS T EM CÃES

RESUMO- O linfoma cutâneo constitui uma forma de linfoma extranodal que afeta inicialmente a pele e/ou anexos cutâneos e que pode apresentar um curso clínico agressivo, avançando a órgãos internos em estádios tardios. O principal imunofenótipo é de células T (LCCT) e representa a forma mais comumente diagnosticada. É esperado que os cães com LCCT desenvolvam resistência à quimioterapia em alguma etapa do tratamento, resultando em tempos curtos de sobrevida. Há uma necessidade por novos alvos de tratamento em cães com LCCT que consigam fornecer melhor expectativa de vida. Em humanos, medicações baseadas em mecanismos epigenéticos têm auxiliado no controle da doença sendo um alvo chave na procura de repostas clínicas mais douradoras em estádios avançados. Neste trabalho descreve-se, nos dois primeiros capítulos relatados, o LCCT e as particularidades descritas na literatura internacional que contrasta com os dados encontrados nesta instituição, assim como, o papel da epigenética na carcinogênese das neoplasias e o mecanismo de modificação de histonas como base para o tratamento. Mais especificamente, no segundo capítulo, foram analisados dados de 21 cães com linfoma cutâneo definido como LCCT de alto grau devido ao tamanho grande das células, à ausência de tropismo na epiderme, ao valor médio encontrado de Ki67 de 63,9% e ao tempo de sobrevida estimado de 31 dias. Características clinico-patológicas foram analisadas para a identificação de marcadores prognóstico, sendo definido as alterações decorrentes pelo linfoma vistas na radiografia como marcador prognóstico negativo independente depois da análise multivariada. No último capítulo relatado, amostras de LCCT foram avaliadas para conhecer o nível de histonas acetiladas (H3Ac e H4Ac) e de histonas deacetilases (HDAC) HDAC1, HDAC2 e HDAC6, as quais encontram-se envolvidas no mecanismo de modificação de histonas em LCCT de humanos. O objetivo desse estudo foi comparar amostras de LCCT com linfonodo normal, pele inflamada e pele normal de cães. Tanto as H3Ac e H4Ac como as HDAC1, HDAC2 e HDAC6 estiveram aumentadas em LCCT quando comparadas com pele normal, de igual maneira encontrou-se que o nível de H3Ac foi estatisticamente menor em linfoma que em linfonodo normal, e que um aumento aberrante de H4Ac e HDAC2 foi constatado em LCCT. Adicionalmente, evidenciou-se que a associação da imunoexpressão de H3Ac, H4Ac e HDAC2 classificou duas populações, as quais apresentaram tempos de sobrevida diferentes (48 dias Vs 22 dias, p=0.06), sugerindo assim um perfil de histonas existente em amostras de LCCT. Este estudo confirmou que o nível de histonas deacetilases em LCCT é maior que em tecidos saudáveis. Futuros estudos são necessários para corroborar nossos resultados e para que futuramente inibidores das HDAC possam ser utilizados em cães com LCCT.

Palavras-chave: acetilação, canino, epigenética, HDAC, linfoma extranodal, oncologia

EVALUATION OF ACETYLATED HISTONES 3 AND 4 AND HISTONE DESACETYLASE 1, 2 AND 6 IN CUTANEOUS T-CELL LYMPHOMA IN DOGS

ABSTRACT- Cutaneous lymphoma constitutes a form of extranodal lymphoma that affects initially the skin and/or adnexal structures and eventually presents an aggressive clinical behavior, causing internal organ infiltration in late-stage disease. The main immunophenotype is T-cell lymphoma (CTCL) and it represents the most common diagnosed type. It is expected that dogs with CTCL develop resistance to chemotherapy at any point during treatment, resulting in short survival times. A need for new therapeutic targets that improve survival expectation in dogs with CTCL is increasing. In humans, therapies based on epigenetic mechanisms helped to control the disease, since epigenetics became a main objective in the search for longer clinical responses in advance-stages. In the first two reported chapters, CTCL is described with particularities of international literature that contrast with data found in this institution, as well as, the role of epigenetics in the neoplasia carcinogenesis and the mechanism of histone modification as a base for treatment. More specifically, in the second chapter, 21 dogs with CTCL were defined as having high grade CTCL due to their large cell size, absence of epithelial tropism, mean value of Ki67 of 63,9% and estimated survival time of 31 days. Clinicopathological characteristics were analyzed for identifying prognostic markers, being the intrathoracic involvement caused by lymphoma seen on thoracic radiography an independent prognostic factor after multivariate analysis. In the last study, samples of CTCL were evaluated to know the level of acetylated histones (H3Ac and H4Ac) and histone deacetylate enzymes (HDAC) HDAC1, HDAC2 and HDAC6, which were involved in the epigenetic mechanism of histone modification in human CTCL. The objective of this study was to compare samples of CTCL in normal lymphnode, inflammatory cells in skin and normal epithelial cells. All markers (H3Ac, H4Ac, HDAC1, HDAC2 and HDAC6) were found to be higher in CTCL than in normal skin, furthermore, the level of H3Ac was statistically lower in CTCL than in normal lymphnode and an aberrant higher level of H4Ac and HDAC2 was confirmed in CTCL. Additionally, the association of the immunoexpression of H3Ac, H4Ac and HDAC2 classified into two the population, having different survival times (48 days Vs 22 days, p=0.06), suggesting that a histone profile exists in the studied population. This study confirmed that the level of histone deacetylases in CTCL are higher than in normal tissues. Further studies are needed to confirm our results and to support new research in HDAC inhibitors in dogs with CTCL.

Keywords: acetylation, canine, epigenetic, extranodal lymphoma, HDAC, oncology

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CHAPTER 1 - General considerations

1. Introduction

The term lymphoma involves a group of diverse diseases that have a common cell of origin but differs among locations and several other aspects including tumor behavior, clinical manifestation, treatment approach and prognosis. In this regard, cutaneous lymphoma (CL) is considered a rare and incurable form of lymphoma that affects multiple species (Kuzel et al., 1991; Fontaine et al., 2009; Fontaine, Heimann and Day, 2011; Miller et al., 2015). In dogs, CL is characterized by the presence of variable cutaneous signs and challenging treatment that finally lead the patient to disease progression and poor prognosis (Fontaine et al., 2009; Rook, 2019). In Brazil, information about this disease is scarce; incidence seems to be higher and survival times shorter than the reported in international literature (Duarte et al., 2016).

Canine CL has been widely reported to respond to chemotherapy, therefore, different types of drugs have been used against this disease (Lemarie and Eddlestone, 1997; De Loremier, 2006; Risbon et al., 2006; Williams et al., 2006; Morges et al., 2014), but mean of survival time does not exceed 6 months according to a recent treatment review (Laprais and Olivry, 2017). In humans, the classical form of CL is called Mycosis Fungoides (MF) and this disease manifests as patches or plaques at initial steps and nodules or systemic dissemination in late stages (Olsen et al., 2007a). Despite the good prognosis of MF in humans, eventually cases of systemic involvement and multiple nodules develop in different areas of the body and systemic treatments based on chemotherapy are combined with a range of new drugs (Li et al., 2012). One type of drugs used in advanced MF are histone deacetylase (HDAC) inhibitors that are approved by the FDA since 2006 (Olsen et al., 2007b; Li et al., 2012). These kind of drugs promote response through histone acetylation that lead the cells to permit the transcription of suppressor genes, resulting in apoptosis and inhibition of tumor proliferation (Lane and Chabner, 2009; Khan and Thangue, 2012).

The use of HDAC inhibitors is rarely reported in veterinary medicine and it is unknown if a positive effect may be expected in advance stages of CL in dogs, as happens in humans. In order to answer this question, the objective of this research is to know if an acetylation profile exist in dogs with CL that can justify the use of HDAC inhibitors in prospective studies. Additionally, it was imperative to report the findings of a more aggressive CL that is evident in the veterinary hospital of the São Paulo State University, UNESP –Jaboticabal.

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2. Introduction

Cutaneous T-cell lymphoma (CTCL) constitute a type of extra-nodal lymphoma originated from malignant T-lymphocytes that exhibit tropism to the skin (Fontaine et al., 2009). In dogs, although rare, represents 3-7% of all lymphomas (Fournel-Fleury et al., 2002) and it is characterized by a highly variable clinical manifestation that include a generalized erythema with scaling and pruritus –known as exfolliative erythroderma- or patches, plaques and solitary or multiple nodules distributed throughout the superficial area of the body or even close to mucocutaneous junctions and in mucosa (Fontaine et al., 2009; Fontaine et al., 2010; Moore and Olivry, 1994). In contrast to the human counterpart, canine CTCL presents a more aggressive course and is considered to have a poor prognosis (Chan et al., 2018).

Two molecular features have been recognized as the main differences between canine and human CTCL: immunophenotype of T-cells and type of T-cell receptor (TCR) (Moore et al., 2009). Immunophenotype of T-cells in dogs with CTCL is characterized by a lack of CD4 expression (CD4-) and a positive expression of CD8 (CD8+). Eventually, a double CD4-CD8- is also described (Moore et al., 2009). In humans, T-cells are commonly CD4+CD8- and only 10-15% of cases are CD4-CD8+ (Willemze et al., 2019). Furthermore, TCR γδ in dogs with CTCL is recognized in 60% of cases of CTCL, while TCR αβ constitutes the 40% remaining (Moore et al., 2009). In human the most described TCR phenotype is TCR αβ, while TCR γδ characterizes a more atypical and less common form of CTCL (Willemze et al., 2019). Although these differences have been highlighted in several studies, it is still unknown whether they can explain the more aggressive behavior of CTCL in dogs. In the most recent and largest study of canine CTCL dogs lived a median of 8 months being treated with

different treatment modalities (Chan et al., 2018), and other authors have described a median of 6 months using only chemotherapy (Risbon et al., 2006; Williams et al., 2006; Laprais and Olivry, 2017).

Treatment of human CTCL is based on multiple modalities, early-stage lesions are treated with topical agents that include corticosteroids, nitrogen mustard, retinoids/rexinoids, Toll-like receptor agonist (imiquimod) and local modalities like phototherapy with psolaren plus ultraviolet A (PUVA), narrowband ultraviolet B (NB-UVB) and total electron beam irradiation (Hermann et al., 1995; Querfeld et al., 2005; Li et al., 2012). Emerging therapies were further developed in advanced-stage CTCL and immunotherapy including monoclonal antibodies and histone deacetylase inhibitors were added to conventional chemotherapy to provide longer responses and survival times in human CTCL (Kim et al., 2007; Olsen et al., 2007; Querfeld et al., 2009; Li et al., 2012). In veterinary, local therapies are not currently suggested because of limited proved efficacy and generalized disease at diagnosis (De Lorimier, 2006). Patients with solitary lesions performed surgically removal and advanced-stage disease are treated based on chemotherapy, retinoid and/or corticosteroids (Fontaine et al., 2009; Chan et al., 2018). Newer therapies are needed for advanced-stage CTCL in dogs.

Histone modifications are considered a crucial epigenetic mechanism that lead to alter gene transcription in cancer cells with no modification of the DNA sequence (Sharma et al., 2010). These modifications occur in only few residues of the histones tails and include acetylation, methylation, phosphorylation and ubiquitination (Fullgrabe et al., 2011). Among these modifications and in a global way, histone acetylation plays an important role in gene transcription, whereas histone deacetylation involves transcriptional repression or silencing (Khan and La Thangue, 2008). These processes are regulated by two enzymes, one that adds acetyl groups to histones called histone acetyl transferase (HAT) and the another one that removes them called histone deacetylase (HDAC). In human CTCL, emerging drugs like HDAC inhibitors are being used since 2008 when the first HDAC inhibitor –Vorinostat- was released as a therapy for advanced-stage CTCL (Marks and Breslow, 2007). Many different HDAC inhibitors have been used in clinical trials in CTCL (Khan and La Thangue, 2012), and although an exact mechanism of action is still unclear (Sardiu et al., 2014; Ding et al.,

2016), it is believed that benefit of these drugs are product of multiple roles of HDAC inhibitors in malignant cells including apoptosis presumably by reactivating silenced genes (Khan and La Thangue, 2012). By inhibiting deacetylation, cancer cells reverse transcriptional silencing of tumor suppressor genes like p53 and pRb resulting in inhibition of tumor growth (Khan and La Thangue, 2012; Lane and Chabner, 2009).

HDACs are overexpressed in several human malignancies (Chen et al., 2015), and some authors have demonstrated their high expression in multicentric lymphoma and CTCL (Marquard et al., 2008; Marqueard et al., 2009). CTCL presents a higher therapeutic response to HDAC inhibitors in advanced-stage CTCL, showing overall responses rates up to 46% (Lopez et al., 2018). In veterinary, dogs with CTCL finally died because of chemotherapy resistance and disease progression (Fontaine et al., 2009), and no other therapies are attempted in advanced-stage.

Due to dysregulation of H3Ac, H4Ac and HDAC1, HDAC2 and HDAC6 in human CTCL (Marquard et al., 2008) and the urgent search for new therapies in dogs with the same disease, we wanted to evaluate the protein level of HDAC and acetylated histones in samples of dogs diagnosed with CTCL and compare it to normal lymphoid tissue, inflammatory lymphocytes and healthy skin. With this research, we proposed to investigate one of the epigenetic mechanisms proved to be involved in human cutaneous lymphoma and make the first step in recognize histone deregulation in canine CTCL.

6. Conclusion

Based on these results, we conclude that a immunoexpression profile of H3Ac H4Ac and HDAC1, HDAC2 and HDAC6 exists in CTCL. We showed that these proteins were statistically higher in CTCL than in normal epithelial cells, demonstrating a different pattern in cancer. The pattern of expression was similar between inflammatory and tumor lymphocytes for all antibodies, except for HDAC6. An acetylation aberrant pattern was observed in CTCL by the demonstration of high levels of H4Ac. By contrast, a low H3Ac levels was a common characteristic in samples of CTCL and dogs expressing high levels of H3Ac presented better survival times. An association among the immunoexpression of HDAC2, H3Ac and H4Ac could define a population with worse survival, and the use of HDAC inhibitors should be considered in dogs with this disease, however, further studies are needed to confirm our results in order to stimulate the use of HDAC inhibitors drugs in canine CTCL.

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