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Universidade Estadual Paulista “Júlio de Mesquita Filho”
Faculdade de Medicina Veterinária e Zootecnia

NEW PREDICTIVE FACTORS IDENTIFIED BY MOLECULAR STUDY IN CHEMORESISTANT HUMAN AND CANINE MAMMARY CARCINOMA CELLS

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Botucatu, SP
September – 2024

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Thesis presented to the Postgraduate Program in Animal Biotechnology to obtain the title of Ph.D.

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“If you have knowledge, let others light their candles in it.”

Margaret Fuller

RESUMO

LAINETTI, P.F. **ESTUDO MOLECULAR PARA IDENTIFICAÇÃO DE NOVOS FATORES PREDITIVOS EM CÉLULAS MAMÁRIAS HUMANAS E CANINAS QUIMIORRESISTENTES**. Botucatu – SP. 2024. 114p. Defesa (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

As afecções mamárias em cadelas ocorrem de maneira muito frequente em animais não castrados e com idade mais avançada. A maioria desses tumores é classificado como maligno e a ocorrência de metástases em linfonodos e órgãos distantes pode chegar a 30%. Os tumores mamários em cadelas compartilham semelhanças com os tumores em mulheres, incluindo sua ocorrência espontânea e maior incidência em indivíduos mais velhos, além de características clínicas e moleculares similares. Genes envolvidos no desenvolvimento do câncer atuam de maneira semelhante em ambas as espécies. Embora a remoção cirúrgica seja o tratamento padrão, a quimioterapia é discutida como opção complementar. No entanto, a resistência ao tratamento é um desafio, podendo estar relacionada a mecanismos genéticos adquiridos ou pré-existentes nos tumores. As mutações genéticas, como a presença de SNPs and CNVs no DNA, podem explicar a falha terapêutica. Este estudo tem como objetivo identificar alterações genéticas associadas à resistência à quimioterapia em células de carcinomas mamários de cadelas, avaliar a presença de SNPs nessas amostras, além de relacionar a expressão dos miR-29b, miR-146b, miR-191 e miR-375 com 36 amostras independentes de tumores mamários em bloco de parafina. Durante a pesquisa, foram estabelecidas culturas celulares de duas amostras, UNESP-CM70 e UNESP-CMR1, que foram aplicadas em camundongos para avaliar sua capacidade tumorigênica. Outras 3 culturas celulares, previamente caracterizadas, também foram utilizadas para os experimentos, as células UNESP-CM1, UNESP-CM4 e UNESP-CM5. A expressão dos genes *MDR1, BCRP, MRP1 e MRP3*, relacionados à resistência à quimioterapia, foi avaliada nas células pela técnica de RT-qPCR. O teste de metabolismo celular (MTT) foi realizado com a doxorrubicina para definir a concentração inibitória mínima (IC_{50}) antes e depois da indução à resistência

terapêutica com a doxorrubicina. O DNA das células, após o tratamento, foi avaliado pela técnica de SNP array. A avaliação dos miRNAs (miR-29b, miR-146b, miR-191, miR-375) foi realizada por RT-qPCR em 36 amostras, divididas em 3 grupos, grau I, grau II e grau III. Apenas a células UNESP-CM70 foi capaz de induzir o crescimento *in vivo*. A IC_{50} foi determinada para cada tipo de célula, no grupo controle e no grupo tratado. As IC_{50} foram UNESP-CM1 CTRL (7.8 μM), UNESP-CM1 DOX (0.21 μM), UNESP-CM4 CTRL (12.54 μM), UNESP-CM1 DOX (2.9 μM), UNESP-CM5 CTRL (0.04 μM), UNESP-CM5 DOX (0.57 μM), UNESP-CM70 CTRL (1.67 μM), UNESP-CM70 DOX (6.3 μM), UNESP-CMR1 CTRL (0.5 μM) e UNESP-CMR1 DOX (0.63 μM). Os resultados da RT-qPCR identificaram que célula UNESP-CMR1 apresentou maior expressão dos genes avaliados em comparação com a outras células. Foram encontrados dois genes relacionados às mutações de SNPs, os genes ALK e o RALGAPA2, que estão associados à progressão, invasão tumoral e metástase. Os miR-29b, miR-146b e miR-375 foram encontradas menos expressos no grupo grau III quando comparado com os grupos grau I e grau II. Os miRNAs podem estar relacionados com genes responsáveis por vias de sinalização PI3K-AKT, de adesão focal e na interação com a matriz extra celular.

Palavras-chave: cão, carcinoma mamário, SNP, resistência quimioterápica, cultivo celular

ABSTRACT

LAINETTI, P.F. **NEW PREDICTIVE FACTORS IDENTIFIED BY MOLECULAR STUDY IN CHEMORESISTANT HUMAN AND CANINE MAMMARY CARCINOMA CELLS**. Botucatu – SP. 2024. 114p. Defesa (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

Mammary disorders in dogs occur very frequently in non-neutered and older animals. The majority of these tumors are classified as malignant and the occurrence of metastases in lymph nodes and distant organs can reach 30% of all cases. Canine mammary tumors (CMTs) share similarities with women's Breast Cancer (BC), including their spontaneous occurrence and higher incidence in older individuals, in addition to similar clinical and molecular characteristics. Although surgical removal is the standard treatment, chemotherapy is discussed as an adjuvant treatment. However, resistance to treatment is a challenge and may be related to acquired or pre-existing genetic mechanisms in tumors. Genetic mutations, such as the presence of SNPs and CNVs in DNA and miRNAs dysregulation can be involved in CMTs and BC resistance. In human BC, several studies have investigated the genetic and epigenic mechanism of chemoresistance. However, little is known about this mechanism in CMTs development and progression. Therefore, this study aimed to identify genetic changes and miRNA dysregulation associated with resistance to chemotherapy in mammary carcinoma cells from dogs and model to human BC. In the first step of this research, five cell lines were used and *MDR1*, *BCRP*, *MRP1*, *MRP2* and *MRP3* gene expression were assessed to evaluate the baseline expression of these multiple drug resistance genes. Then, these cells were subjected to an induced chemoresistance protocol using doxorubicin. The cell DNA was collected prior and after inducing treatment and subjected to a SNP microarray platform. In this step, we established a standard protocol for inducing MCTs cancer cell lines chemoresistance and identified several SNPs related to chemoresistance in vitro. Only UNESP-CM70 cells were able to induce growth *in vivo*. The IC_{50} was determined for each cell type, in the control group and in the treated group. The IC_{50}s were UNESP-CM1 (CTRL = 7.8 μM; DOX = 0.21 μM),

UNESP-CM4 (CTRL = 12.54 μM; DOX = 2.9 μM), UNESP-CM5 (CTRL = 0.04 μM; DOX = 0.57 μM), UNESP-CM70 (CTRL = 1.67 μM; DOX = 6.3 μM), UNESP-CMR1 (CTRL = 0.5 μM; DOX = 0.63 μM). Two genes related to the SNP mutations were found, *ALK* and *RALGAPA2*, which are associated with tumor progression, invasion, and metastasis. Then, we evaluated miRNAs (miR-29b, miR-146b, miR-191, miR-375) RT-qPCR in 36 CMTs tissue samples. The miR-29b, miR-146b and miR-375 were found to be downregulated in group grade III when compared to the others groups. These miRNAs may be related to genes responsible for PI3K-AKT signaling pathways, focal adhesion and interaction with the extra-cellular matrix. It was possible to conclude that SNP mutations in *ALK* and *RALGAPA2* genes were identified during CMT chemoresistance induction and miRNA downregulation was associated with high grande CMTs.

Keywords: dog, mammary carcinoma, SNP, chemotherapy resistance, cell culture

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LIST OF ABBREVIATIONS, ACRONYMS and SYMBOLS

ABC: *ATP-binding cassette*
BCRP: Breast cancer resistant protein
***BCRP*:** Gene encoding breast cancer resistance protein
cDNA: Complementary DNA
CNV: *Copy number variation*
DMEM: *Dulbecco's Modified Eagle Medium*
DMSO: Dimethylsulfoxide or Dimethyl Sulfoxide
DNA: Deoxyribonucleic acid
ER: Estrogen receptor
HER2: Epidermal growth factor receptor 2
MDR1: P-glycoprotein
***MDR1*:** Gene encoding P-glycoprotein
MRP1: Multidrug resistance protein 1
***MRP1*:** Gene encoding multidrug resistance protein 1
MRP2: Multidrug resistance protein 2
***MRP2*:** Gene encoding multidrug resistance protein 2
MRP3: Multidrug resistance protein 3
***MRP3*:** Gene encoding multidrug resistance protein 3
MTT: *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* ou ensaio de avaliação da atividade metabólica celular
PARP: *Poly (ADP-ribose) polymerase*
PBS: *Phosphate-bufferid saline*
PCR: *Polymerase Chain Reaction*
PET: Positron emission tomography
PR: Progesterone receptor
RNA: Ribonucleic Acid
RT-qPCR: *Real Time quantitative PCR*
FBS: Fetal Bovine Serum
SNP: *Small nucleotide polymorphism*

SYMBOLS

°C: Degrees Celsius

h: Time

%: Percent

µM: Micromolar (10-6 mol/L)

µL: Microliter

min: Minutes

mL: Milliliter

L: Liter

s: Second

nmol: Nanomole

SUMARY

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CHAPTER 1

1. INTRODUCTION

Mammary neoplasms represent approximately 50% of tumors identified in female dogs, the majority of which are malignant (SORENMO et al., 2003; SLEECKX et al., 2011). Tumors in female dogs have similarities to tumors in women, including their spontaneous occurrence and increased incidence with age (SALAS et al., 2015). Furthermore, features such as disease course, clinical stages, lymph node invasion, and important molecular markers in carcinogenesis are similar in both species (PEÑA et al., 2014; ABDELMEGEED; MOHAMMED, 2018; NGUYEN et al., 2018).

Genes involved in cancer development act similarly in human and canine mammary neoplasms (SHEARIN et al., 2010; KASZAK et al., 2018). In addition, both species are exposed to similar carcinogens due to their shared environment which results in similar risk factors and molecular alterations. Several types of neoplasia are also found in both humans and dogs including lymphoma, lung carcinoma, and osteosarcoma (YU; CHEAH, 2017).

Chemotherapy as an adjuvant treatment for mammary tumors in dogs is still a subject of debate since surgical removal of the tumors is considered the standard treatment (CASSALI et al., 2020; HORNFELDT et al., 2023). Although there are different chemotherapy protocols for dogs with mammary tumors, there is no standard protocol with proven efficacy in tumor remission, adjuvant therapy, and increased survival, making the use of chemotherapy for these tumors up for debate (TRAN, MOORE, FRIMBERGER, 2016; TURNA et al., 2023).

Lack of response to treatment can occur due to several factors including acquired or pre-existing mechanisms. Acquired mechanisms are usually a result of exposure to chemotherapy drugs, whereas pre-existing mechanisms refer to tumor characteristics that can be altered by the tumor microenvironment (LOVITT et al., 2018; BUKOWSKI et al., 2020). In women, approximately 15% of patients with breast cancer develop metastases within three years after diagnosis of the primary tumor, and metastases are the leading cause of death in these cases (WEIGELT et al., 2005). Although there is an initial response to chemotherapy in women and dogs some mammary tumors develop resistance to the most commonly used drugs highlighting the need for new therapeutic options (KLEIN et al., 2019).

Understanding the mechanisms of resistance to chemotherapy is crucial

to determine when and how to apply antineoplastic therapy. However, knowledge on this topic in dogs is limited. Chemotherapy is recommended for animals with more aggressive tumors, metastases or tumor recurrences, but its efficacy is still uncertain since many patients have recurrences after treatment (SIMON et al., 2006; LEVI et al., 2016).

Morphological, behavioral, and clinical similarities between mammary tumors in dogs and women in addition to the high genomic homology between the species make these tumors a good model for the study of mammary carcinoma in women. Furthermore, with the discovery of new drugs, it is possible to initially test them in dogs which serve as preclinical models for women. Therefore, both groups benefit from comparative studies. Women also face challenges related to chemotherapy resistance and adjuvant therapies currently target molecular targets. However, even with a focused therapy for these targets some tumors still develop resistance and become metastatic. For that reason, the discovery of new molecular targets is important to provide more treatment options (HERNANDEZ; GONZALEZ, 2013; GU et al., 2016).

On that account, this study aimed to identify and validate genetic alterations associated with resistance to chemotherapy in mammary carcinoma cells from dogs. After validating these alterations, we will investigate the potential of different therapeutic targets in cultures of mammary carcinoma from dogs, to identify drugs that can be associated with the treatment of chemotherapy-resistant tumors. It is worth mentioning that there is a lack of information on chemotherapy resistance in canine mammary tumors except for an article published by Zambrano-Estrada et al. (2018) which relates the use of doxorubicin in combination with molecular iodine (I2) to increased survival in dogs with mammary tumors. In our searches in national and international databases, we found no other articles that describe comparative data on chemotherapy resistance between human and canine mammary tumors.

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Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

Author Contributions

Conceptualization (C.L.; P.F.L.; V.G.), data curation (P.F.L.; A.F.L.F.), formal analysis (P.F.L.; A.F.L.F.; C.L.), funding acquisition (C.L.; F.C.; V.G.), investigation (P.F.L.; A.F.L.F.; C.Z.; E.S.), methodology (C.L.), project administration (P.F.L.), supervision (V.G.; C.L.; C.E.F.A.), writing – original draft (P.F.L.), writing – review & editing (V.G.; C.L.; F.C.; C.E.F.A.; A.F.L.F.; C.Z.).

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