S U M M A R Y

STUDIES REGARDING THE DETECTION OF CIRCULATING TUMOR CELLS IN THE BLOOD OF DOGS WITH MAMMARY CARCINOMA (*CANIS LUPUS FAMILIARIS*)

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Canine mammary tumours are a major health problem and the most commonly diagnosed tumour in females, 50-80% of which are malignant. Research in the field of veterinary oncology is growing, focusing on the development of new techniques for early diagnosis and the discovery of new biomarkers that can be used to detect tumours or to assess prognosis and treatment efficacy.

The research included in this study was carried out in the Pathology Laboratory of the Faculty of Veterinary Medicine, University of Agronomic Sciences and Veterinary Medicine, Bucharest, and included antemortem investigations in female canines and studies on tumour cell lines. The PCR examination was also carried out at the Faculty of Veterinary Medicine and the immunofluorescent examination at the National Institute for Medical and Military Research and Development "Cantacuzino". Cell line tests were performed at the National Institute for Research and Development "Victor Babes" Bucharest. The design and manufacture of the microfluidic device was carried out at the National Institute for Research and Development in Microtechnology - IMT Bucharest.

Thesis structure. The PhD thesis "Studies regarding the detection of circulating tumor cells in the blood of dogs with mammary carcinoma (*canis lupus familiaris*)" is structured according to legal requirements in two main parts. *Part I: Bibliographic study* comprises 34 pages, representing 27.6% of the work. This part is structured in four chapters and presents the anatomy, histology and physiology of the mammary gland in the dog, the tumour process and metastasis of mammary gland tumours and the detection and clinical implications of circulating tumour cells. *Part II: Personal research* is structured in five chapters and includes the aim and objectives of the research, the study material and methods used for diagnosis, for the development of the microfluidic system and for its testing, evaluation of the presence of EpCAM and CRYAB molecules in mammary carcinoma, description of the technological flow for the development of the

microfluidic device, experiments for testing the device on tumor cell lines and experiments for testing the device on patient samples. This part comprises 89 pages, representing 72.4% of the paper.

Part I: Bibliographical study

Chapter I - Anatomy, histology and physiology of the mammary gland in female dogs provides information on the appearance, morphology and normal physiology of the mammary gland in female dogs. This information is imperative in order to be able to observe and distinguish the pathological changes that occur with the development of a tumour at this level.

Chapter II - Mammary tumour process contains generalities about the tumour process and describes the mechanisms of carcinogenesis. The chapter presents the classification of mammary tumour types and the histological description of malignant mammary tumours of epithelial origin. Information on the frequency, staging and prognosis of mammary tumours in dogs is also presented.

Chapter III - Metastasis of mammary gland tumours in the female presents generalities about the metastatic process and the pathogenesis of metastasis. As this thesis proposes the detection of circulating tumour cells, this chapter is particularly important for understanding how tumour cells reach the bloodstream.

Chapter IV - Circulating tumour cells, detection and clinical implications provides information on the general characteristics of circulating tumour cells and data on the current status of the use of circulating tumour cells in veterinary medicine. The chapter presents markers used for the detection of circulating tumour cells in dogs and devices and methods for the detection of CTCs in human and veterinary medicine. The last section of the chapter presents the clinical implications in human and veterinary medicine.

Part II: Personal research

Chapter V - General materials and methods used for the detection of circulating tumour cells includes information on the animals studied, diagnostic methods, molecular biological techniques for the detection of tumour marker molecules on the cell's surface, tumour cell lines used to test the functionality of the device and the technological process used to manufacture the microfluidic device.

The research conducted in this study took place from 2016-2023 on 12 dogs from which 9 tumor tissue samples and 3 blood samples were collected. The research activities included specific activities for the diagnosis and characterization of tumor markers, activities for the microfabrication of a microfluidic system with an integrated electrochemical sensor, and activities for the detection of circulating tumor cells in blood samples from dogs with mammary carcinoma.

To demonstrate the functionality of the electrochemical sensors developed in this study, three tumor cell lines were used: human colon adenocarcinoma cell line SW-403 (catalog no. 87071008), HT-29 (catalog no. 91072201) and MCF-7 breast cancer cells

(HTB-22-ATCC). The device was also tested using 3 blood samples from mammary carcinoma patients.

The methods used for diagnosis and molecular biology tests consisted of cytopathological examination, histopathological examination, immunofluorescence examination and Polymerase Chain Reaction examination.

The methods used for the development of the microfluidic system and for the detection of circulating tumor cells were: microfabrication, scanning electron microscopy (SEM), Raman spectroscopy, electrochemical measurements, growth of vetical graphene, synthesis of gold nanoparticles, immobilization of anti-EpCam antibodies and obtaining PDMS and encapsulation of the electrochemical sensor.

Chapter VI - Detection of EpCAM and CRYAB molecules in canine mammary carcinoma the EpCAM molecule and 7 tumor mRNA markers namely CLDN7, CRYAB, ELF3, SLC1A1, ATP8B1, EGFR and F3 expressed in canine mammary tumors with different histotypes were studied. Fresh tissue samples were analyzed using two different techniques to identify the molecules of interest. To detect EpCAM in fresh tissue samples, we used immunofluorescence methods after freezing the tissue and cutting it with a cryotome. To detect mRNA we used real-time PCR detection.

EpCAM molecule was detected in 5 fresh tumor tissue samples from the same tumor mass that was used for diagnosis by histopathological examination. Tumor fragments of 2 x 2 cm^2 were used and embedded in Tissue-Tek® O.C.T. Compound and frozen at -80^oC. Immunofluorescence examination was performed by displaing 5 µm cryosections of tumor tissue on glass slides using the cryotome. An indirect immunofluorescence method was used using rabbit polyclonal anti-EpCAM antibodies as primary antibody and goat anti-rabbit fluorescent antibodies as secondary antibodies.

Of the seven markers we proposed for qPCR detection only CRYAB was detected in the 9 samples analysed. The mean quantification cycle (Cq) for samples in which the CRYAB marker was detected was 11.93. Comparing this number with the mean Cq of actin B which was 20.92, we can extrapolate that there was an increase in CRYAB mRNA marker activity in the tumour samples. RNA was extracted from fresh 15 mg tumour tissue samples by the 'purification of total RNA from animal tissue' protocol using the RNeasy Mini kit. This method is used to enrich mRNA as only RNA molecules longer than 200 nucleotides are trapped in the silicon membrane of the kit, the rest of the RNA molecules such as 5.8SrRNA, 5S rRNA and tRNAs being smaller are selectively excluded. The final sample volume was 50 µl. Detection of mRNA markers was performed using the real time-PCR (qPCR) method.

EpCAM was detected in five different tumor tissues from dogs with different types of mammary carcinomas using immunofluorescence techniques. CRYAB was also detected in nine different tissue samples from dogs with different types of mammary carcinomas using real-time PCR examination. More aggressive types of mammary

carcinomas expressed higher EpCAM and CRYAB values. CRYAB can be used as a marker for the diagnosis of mammary carcinomas and to assess tumour aggressiveness.

Chapter VII - Electrochemical sensor fabrication describes the technological processes by which two types of electrochemical sensors for the detection of circulating tumour cells were obtained. These two sensors were individually encapsulated in a polymer called polydimethylsiloxane (PDMS) to obtain microluximide devices capable of analysing blood samples in a continuous flow to avoid sample loss or contamination.

The devices are based on a 4-inch silicon wafer on which, by optically transferring the geometry of the masks onto the silicon wafer coated with a photoresist substrate, a series of 23 electrochemical sensors with gold electrodes were made. These, by adding additional technological processes, were improved by coating the interdigitated working electrodes with a vertical graphene layer. Vertical graphene is a state-of-the-art 3D nanocarbon material designed to improve sensor performance. Furthermore, the vertical graphene has been decorated with gold nanoparticles (AuNP) which are designed to increase the surface area of the graphene, thus the current and sensitivity of the sensor, and to increase the specificity of detection by attaching more antibody molecules to the same sensing surface.

This type of sensor is an "In House" type sensor with unique design and geometry designed and developed at the National Institute for Research and Development in Microtechnology - IMT Bucharest. The sensor has a size of 25x10mm^2 and features an auxiliary electrode, a reference electrode and an interdigitated working electrode with 64 interdigitated pairs with a width of 20 µm and a gap of 10 µm.

The development of a new method of encapsulating electrochemical sensors using 3D printing was also demonstrated in this chapter. The encapsulation of the sensor in PDMS, transforms a simple sensor into a complex microfluidic device that allows for the complete analysis of blood samples in a controlled manner minimizing sample loss and contamination.

Chapter VIII - Vertical graphene-based biosensor for assessing the dielectric signature of tumour cells describes the first stage of electrochemical sensor testing. Because the heterogeneity of circulating tumour cells is high and their number in a blood sample is unknown, samples with known concentrations of tumour cells from standardised and morphologically characterised tumour cell lines were used to make the test step scientifically relevant.

The dielectric properties of tumor cells were evaluated using an EIS-based biosensor. The sensor was tested using three different tumor cell lines: human breast cancer tumor cells (MCF-7), colorectal adenocarcinoma cell line SW-403 and colorectal adenocarcinoma cell line HT-29. The novelty of the approach consists in using a vertical graphene-based electrochemical biosensor. We demonstrated that by using the developed sensor we could differentiate between three different types of tumor cell lines based on the specific dielectric signature of each cell line.

The biosensor was able to detect 100 cells mL^{-1}. After analizing the obtained data we concluded that the cells of the HT-29 tumor cell line have a much larger membrane area and lower capacitive reactance compared to the cells of the SW-403 cell line. Because the HT-29 cells have a higher membrane capacitance they store a greater number of electrical charges, which leads to higher conductivity and to lower resistance to charge transfer. The membrane microvilli contain a very high concentration of lipid reafts, especially cholesterol-glycosphingolipids. Thus, we can deduct that the HT-29 cells have more lipid rafts than SW-403 cells. SW-403 tumour cells tend to adhere and form large cell clusters. This fact is validated by EIS measurements using the developed sensor. MCF-7 are large and adherent cells with a size of 20-25 μm. The results show that they have lower charge transfer resistance and higher conductivity and permittivity than the other two studied tumor cell lines.

Chapter IX - Detection of circulating tumour cells in canine mammary carcinoma presents the second stage of testing the microfluidic device both with samples with known concentrations of tumour cells from the MCF-7 breast tumor cell line used as a model for detection of circulating tumour cells and with blood samples from mammary carcinoma patients in which detection and capture of possible circulating tumour cells in the blood stream was attempted.

The tests were done using one of the sensors developed in Chapter VII, an EIS sensor encapsulated in PDMS. This consists of an interdigitated gold electrode biosensor functionalised with anti-EpCAM antibodies capable of capturing and analysing the dielectric properties of captured CTCs. Also, a novel metastatic marker was also evaluated using anti-CD36 antibodies. The device was tested using the breast cancer tumor cell line MCF-7 as a circulating tumor cell model. The device was also tested using three blood samples from dogs with mammary carcinoma. Whole blood was lysed to remove red blood cells and the resulting samples were run through the microfluidic device. CTCs binding was evaluated based on impedance change.

We demonstrated the sensitivity of the microfluidic device by detecting only 3 MCF-7 cells on the electrode surface. Also, by using the device we detected CTCs in blood samples from mammary carcinoma patients. CTCs are quantified based on the increase of the impedance which is directly proporsional with the number of captured cells. The Bode diagram shows the dielectric characteristics of captured CTCs.