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MOLECULAR CHARACTERIZATION OF DOXORUBICIN RESISTANCE AND CANCER STEM CELLS IN A CANINE MAMMARY ADENOCARCINOMA CELL LINE

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By

KABIRU SAHABI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This thesis is dedicated to my family in recognition of their outstanding contributions to my academic endeavours

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Gayathri Thevi Selvarajah, PhD
Faculty : Veterinary Medicine

Canine mammary gland tumour (CMT) is the commonest neoplasm affecting the female dog, with a prevalence of 39% in Malaysian female dogs. Metastasis to the lung is a common cause of death in the affected dogs. Cancer stem cells (CSCs) are small proportion of cancer cells that are able to initiate tumours, metastasis and facilitate recurrence due to properties such as self-renew, longevity, resistance to radiotherapy and chemotherapy, undergo a dormant state, ability to differentiate and evade apoptosis. Drug resistance in cancer cells, either acquired or inherent is a major cause of failure of many forms of anti-cancer chemotherapy mostly due to the expression of drug efflux genes called ATP-binding cassettes (ABC) and other factors. MicroRNAs (miRNA) are short non-coding RNA that can inhibit the translation of messenger RNA (mRNA) to protein in multicellular organisms; where majority of mRNAs are predicted to be under the control of miRNA.

The objective of this study was to develop a doxorubicin resistant CMT cell line and determine its *in vitro* and *in vivo* (tumour induction capabilities in mouse models) characteristics, and to isolate and characterize (mRNA expression and miRNA transcription) CSCs from the cell line and its doxorubicin-sensitive original cells.

Doxorubicin-resistant CMT-Star cells were developed from CMT-Stylo cells by exposing the cells to increasing concentrations of doxorubicin from 10 nM to 100 nM over 5 months. *In vitro* characterization was performed using the MTT assay, anchorage independent growth, AO/PI assays; and *in vivo* tumorigenic and metastatic properties in NOD/SCID mouse models. Quantitative real-time PCR (QPCR) was used to evaluate the expression of selected drug resistant genes in CMT-Star cells. Allophycocyanin

conjugated CD44 and R-Phycoerythrin conjugated CD24 antibodies were used to sort putative CSC from the 2 cell lines. QPCR was used to determine the expression of stem cell marker aldehyde dehydrogenase in the sorted cells. Gene expression profiling of the two cell lines and sorted CSC was done using a 44K canine specific gene expression microarray by Agilent Technologies®. The miRNA profiling was done using Agilent SurePrint® custom canine specific miRNA platform. Signalling pathways, biological and cellular processes involved in drug resistance and CSC was explored using GeneSpring® and Database for Annotation, Visualization and Integrated Discovery (DAVID). Hierarchical clustering was performed on the gene expression profiles and Venn diagrams used to show overlapping genes in the datasets.

The expressions of *ABCB1* and *ABCG2* were significantly increased in CMT-Star cell line. Both cell lines developed tumours in NOD/SCID mice. The expression of 785 genes and 14 miRNAs were altered in CMT-Star cells. Downregulating Plasminogen (*PLG*), plasminogen activator urokinase (*PLAU*); while upregulating transforming growth factor beta receptor 3 (*TGFβR3*) and *ABCB1* makes CMT-Star cells less proliferative, less invasive and more resistant to chemotherapeutic drugs. The expression of 309 genes and 6 miRNAs were altered in CMT-Stylo CSC, while the expression of 206 genes and 14 miRNAs were altered in CMT-Star CSC. The upregulation of *TGFβR 3, IL-6*, nuclear receptor subfamily 4, Wnt Signalling, EGFR1 Signalling were observed in the two CSC populations, making the CSC in this study quiescent, self-renewing, tumour initiating, able to avoid detection by the immune system and drug resistant. Novel miRNAs, yet to be characterized in cancer were also identified with changes in transcription in various comparisons that are yet to be explored.

In conclusion, this study has profiled the miRNA transcription and mRNA expression in the sub population of cells with CSC marker expression from canine mammary adenocarcinoma cells. The identified miRNAs and mRNAs regulate factors and pathways that maintain the doxorubicin resistant and CSC phenotypes, and require further research to investigate their potentials as therapeutic targets to facilitate development of new therapeutic strategies based on these novel miRNA and mRNA targets.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERINCIAN MOLEKULAR SEL-SEL KANSER INDUK ADENOKARSINOMA MAMARI KANIN DAN SEL-SEL YANG RINTANGAN KE ATAS DRUG DOXORUBICIN

Oleh

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Tumor kelenjar mamalia (CMT) adalah neoplasma yang paling lazim pada anjing betina, didapati pada jangkaan 39% daripada anjing-anjing betina di Malaysia. Metastasis ke peparu adalah penyebab utama kematian pada kanin yang terjejas. Sel-sel stem kanser (CSC) merangkumi bahagian kecil dalam populasi sel kanser keseluruhan yang mempunyai kelebihan dari segi dapat memulakan pembentukan tumor, metastasis dan memudahkan pertumbuhan semula selepas surgeri disebabkan ciri-ciri seperti keupayaan untuk memperbaharui sel, meningkatkan jangka hayat sel kanser, memperoleh daya tahan terhadap radioterapi dan kemoterapi, menjadi "dormant" (tidak aktif) dalam kanser dan berupaya untuk membezakan dan mengelak daripada proses apoptosis. Rintangan drug dalam sel kaser, samada diperoleh atau inheren adalah punca utama kegagalan pelbagai kemoterapi anti-kanser selalunya disebabkan oleh gen efluks dinamakan ATP-binding cassettes (ABC) dan faktor-faktor lain. MikroRNA (miRNA) adalah RNA pendek tidak-pengkodan yang boleh merencatkan translasi messenger RNA (mRNA) ke protein dalam organisma multiselular; dimana kebanyakan mRNA adalah dijangkan dibawah pengawalaturan miRNA.

Objektif penyelidikan ini adalah untuk membangunkan CMT sel yang rintang terhadap drug doxorubicin dan menentukan ciri-ciri in vitro dan in vivo (keupayaan membentuk tumor dalam model tikus), dan mengasingkan dan menentukan pencirian (ekspresi mRNA dan transkripsi miRNA) CSC dari sel dan sel asal yang sensitif terhadap doxorubicin

Sel yang mempunyai ketahanan drug doxorubicin (CMT-Star) telah dihasilkan dari CMT-Stylo dengan mendedahkan sel kepada kepekatan doxorubicin yang meningkat dari 10nM ke 100nM selama 5 bulan. Pencirian in vitro telah ditentukan melalui asai MTT, pertumbuhan bebas tambatan, asai AO/PI, dan in vivo tumorigenic dan sifat-sifat metastatic model tikus NOD/SCID. Kuantitatif real-time PCR (QPCR) telah digunakan untuk menentukan ekspresi mRNA terpilih dari gen-gen ketahanan drug untuk mengesahkan sifat-sifat ketahanan drug dalam sel CMT-Star. Marker permukaan sel, CD44 terkonjugat allophycocyanin dan antibody CD24 terkonjugat R-Phycoerythrin telah digunakan untuk mengasingkan yang disangka CSC. QPCR digunakan untuk menentukan ekspresi marker aldehyde dehydrogenase sel induk dari sel yang yang telah diasingkan. Profil ekpresi gen daripada dua sel dan CSC yang telah terasing menggunakan gen spesifik 44K kanin microarray dari Agilent Technologies®. Penyusuk miRNA mengunakan Agilent SurePrint® miRNA spesifik kanin yang ditempah dengan entiti 475 miRNA dan ekspresi mRNA sasaran yang telah dikenalpasti mempunyai fenotip ketahanan doxorubicin dan dalam CSC. Laluan pengisyaratan, proses biologikal dan selular yang terlibat dalam ketahanan drug dan CSC telah diteroka menggunakan GeneSpring® dan Database for Annotation, Visualization and Integrated Discovery (DAVID). Penggugusan berhierarki telah dilaksanakan pada profil ekspresi gen dan diagram Ven untuk menunjukkan gen-gen yang bertindih dalam set-set data.

Ekspresi gen *ABCB1* dan *ABCG2* meningkat secara signifikan dalam sel CMT-Star. Kedua-dua jenis sel tersebut membentuk tumor dalam tikus NOD/SCID. Sebanyak 785 gen dan 14 miRNA didapati berbeza dalam CSC terisolasi daripada CMT-Star. Sejumlah 309 gen telah diekpreskan dalam CSC dari CMT-Stylo, melibatkan pengawalaturan menaik *Interleukin 33, TGFBR 3, nuclear receptor subfamily 4* dan pengawalaturan menurun bagi p*lasminogen (PLG), Plasminogen activator urokinase (PLAU)* dan *interleukin 13 receptor, alpha 2*. Penemuan dalam CMT-Star ini menyebabkan sel kurang proliferative, kurang invasif dan lebih rintang terhadap drug kemoterapi. Sejumlah 206 gen telah diekpreskan dan 14 miRNA telah terubah dalam CSC dari CMT-Star. Keseluruhannya, laluan-laluan yang telah aktif dalam sel kanser induk menyumbang kepada proses onkogenik, pengendalian fenotip sel induk serta terlibat dalam survival sel tumor dan pemproliferatan dan pembentukan kanser. MikroRNA yang novel dijumpai melalui analisis dalam kajian ini di mana mikroRNA tersebut masih belum dikaji sepenuhnya dan peranan mereka masih belum diketahui.

Kesimpulannya, sel kanser induk dari CMT-Stylo dan CMT-Star menunjukkan beberapa perbezaan ekspresi gen yang terlibat dalam pengendalian fenotip sel induk, ketahanan drug dan survival. Kaedah penyelidikan ini berjaya mencirikan ekspresi mikroRNA dan mRNA dalam populasi CSC dari sel adenokarisinoma mamari kanin. Dengan penemuan ini, adalah penting untuk meneruskan penyelidikan untuk memahami patobiologi dan mekanisma ketahanan drug serta membantu pembangunan penyelidikan berdasarkan terapeutik sasaran miRNA dan mRNA.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µL	Microliter
µM	Micro molar
5FU	5-fluorouracil
AACR	American Association for Cancer Research
ABCB1	ATP-binding cassette sub-family B member 1
ABCB5	ATP-binding cassette sub-family B member 5
ABCG2	ATP-binding cassette sub-family G member 2
ABCC1	ATP-binding cassette sub-family C member 1
ABL1	Abelson murine leukemia viral oncogene homolog 1
ABC	ATP binding cassette
AIB1	Amplified in breast cancer 1
ALDH	Aldehyde dehydrogenase
ALL	acute lymphoblastic leukemia
ANKRD1	ankyrin repeat domain 1
ATP	Adenosine triphosphate
AO	Acridine Orange
APC	Allophycocyanin
bFGF	basic fibroblast growth factor
Bcl-2	B-cell lymphoma 2
BCRP 1	breast cancer resistance protein 1
BME	Beta marcaptoethanol
BPI	bactericidal/permeability-increasing protein
CA3	carbonic anhydrase 3

CALML5	calmodulin like 5
cAMP	Cyclic adenosine monophosphate
CCL5	chemokine (C-C motif) ligand 5
CENPJ	centromere protein J
cGMP	Cyclic guanosine monophosphate
CMT	Canine mammary gland tumour
CSCs	Cancer stem cells
CD	Cluster of differentiation
CDK9	Cyclin-dependent kinase 9
cDNA	Complementary deoxy ribonucleotide
cFLIP/FLAME-1	FADD-like anti-apoptotic molecule 1
ChIP	chromatin immunoprecipitation
CLDN1	claudin-1
CMICs	cancer metastasis-initiating cells
COMeT	Comparative Medicine and Technology Unit
COP1 E3	COP1, E3 ubiquitin ligase
CP	Ceruloplasmin
CT	Cycle threshold
CTA	Cancer testis antigen
CTSA	cathepsin A
Cy3	Cyanine-3
CYBB	cytochrome b-245, beta polypeptide (chronic granulomatous disease)
dH_2O	Distilled water
DISC	death-inducing signalling complex

DKK3	Dickkopf-3
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	deoxy ribonucleotide
DPD	dihydropyrimidine dehydrogenase
DUOX1	Dual oxidase 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ERCC1	excision repair cross-complementing 1 protein
ESA	epithelial-specific antigen
EST	Expressed Sequence Tags
FACS	Fluorescence-activated cell sorting
FBN1	fibrillin 1
FBS	foetal bovine serum
FC	Fold change
FDA	food and drug administration of the United States
FDR	False discovery rate
FFPE	formalin fixed and paraffin embedded
FGFR3	Fibroblast growth factor receptor 3
FBXO31	F-box protein 31
FRZB	frizzled-related protein
FWER	Family-wise error rate

GAPDH	Glutaraldehyde phosphate dehydrogenase
GBF1	guanine nucleotide exchange factor 1
GBF1	Golgi brefeldin A resistant guanine nucleotide exchange factor 1
gDNA	Genomic deoxy ribonucleotide
GSTO	glutathione S-transferases
H3	Histone 3
HER-2	human epidermal growth factor receptor-2
HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5
HH	Hedgehog
HIF-1	hypoxia inducible factor 1
HSPG	heparan sulphate proteoglycans
IACUC	Institutional Animal Care and Use Committee
IC_{50}	Inhibitory concentration 50
IFI44L	interferon-induced protein 44 like
IFNG	interferon
IL	Interleukin
IL13RA2	interleukin 13 receptor, alpha 2
INK4	inhibitor of Cyclin-Dependent Kinase 4
IVC	individually ventilated cages
KSFM	keratinocyte serum-free medium
LGR5	leucin-rich repeat-containing G protein-coupled receptor 5
LGALS9	lectin, galactoside-binding, soluble, 9
LIN28A	lin-28 homolog A

LRP	lung resistance-related protein
MACS	magnetic-activated cell sorting
MAPK	mitogen activated protein kinase
MCL1	myeloid Cell Leukemia Sequence 1
MDCK	mardin-Darby canine kidney cells
MGST1	microsomal glutathione S-transferase 1
MHC	major histocompatibility complex
miRNA	microRNA
MIMAT	miRNA matching
MMP	matrix metallo proteinases
MRP	multidrug resistant protein
mRNA	messenger ribonucleotide
MSD	membrane spanning domain
MSI1	musashi homolog 1
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MX1	MX dynamin-like GTPase 1
MYLK	myosin light chain kinase
NANOG	nanog homeobox
NBD	nucleotide binding domain
NDST1	GlcNAc N-deacetylase/N-sulfotransferase-1
NER	nucleotide excision repair
NESTIN	neuroectodermal stem cell marker
NF-kB	nuclear factor kappa B
nM	nano molar

NOD/SCID	non-obese diabetic/severe combined immunodeficient
NOTCH1	Notch Homolog 1, Translocation-Associated
NR4A2	nuclear receptor subfamily 4, group A, member 2
NR1H4	nuclear receptor subfamily 1, group H, member 4
OS	Osteosarcoma
p53	protein 53
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCD4	programmed cell death 4
PDK4	Pyruvate dehydrogenase kinase, isozyme 4
P-gp	P-glycoprotein
PE	Phycoerythrin
PI	Propidium iodide
PKD1	polycystin 1, transient receptor potential channel interacting
PLA2G1B	phospholipase A2 group IB
PLAU	Plasminogen activator Urokinase
PLG	Plasminogen
PLGA	Polylacitde-co-glycolide
PMEA	2-phosphonylmethoxyethyl adenine
PTCH	Patched
PTEN	Phosphatase and tensin homolog
QPCR	quantitative real time polymerase chain reaction
QSAR	quantitative structural activity relationship
RIN	RNA integrity number
RISC	RNA induced silencing complex

RLD	RING-finger-like domain
RNA	Ribonucleotide
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
RORA	RAR related orphan receptor A
RPS19	Ribosomal protein subunit 19
SD	standard deviation
SEA	Single Experiment Analysis
SGSH	N-sulfoglucosamine sulfohydrolase
SH2B1	SH2B adaptor protein 1
SIP1	Smad-interacting protein 1
SIRT 1	Sirtuin 1
Oct4	Octamer-binding transcription factor 4
SPF	specific pathogen free
TAGLN	Transgelin
TBP	Tata binding protein
TCR	T-cell receptor
Tcl1	T-Cell Leukemia/Lymphoma 1
TET	ten-eleven translocation
TFPI2	tissue factor pathway inhibitor 2
TGF-β	transforming growth factor beta
TGFβR	transforming growth factor, beta receptor
TIC	tumour initiating cells
TM4SF20	transmembrane 4 L six family member 20
TNBC	triple negative breast cancer

TNC	tenascin C
TNF-alpha	tumour necrosis factor alpha
TPM-1	tropomyosin-1
UVH	University Veterinary Hospital
VEGF	Vascular Endothelial Growth Factor
XPO5	exportin 5
ZEB1	zinc finger E-box-binding homeobox 1
ZNFX1	zinc finger NFX1-type containing 1

CHAPTER 1

INTRODUCTION

Canine mammary gland tumour (CMT) is the commonest neoplasm affecting the female dog, with a prevalence of 39% in Malaysian female dogs (Sahabi *et al*., 2015). The prevalence of CMT in female dogs is about 4-fold that of breast cancer in women (Cullen, 2002). The disease in the two species share similar epidemiologic, pathologic and prognostic characteristics. CMTs are commonly diagnosed based on histopathology and the primary treatment is surgical resection of the tumour followed by adjunctive therapies to prevent metastasis by targeting the micrometastases in the circulation. This is done typically with cytotoxic chemotherapies such as doxorubicin, cyclophosphamide, 5-fluorouracil and vincristine, whether in single- or multi-agent protocols. However, some CMT variants will respond poorly to these agents (Honscha *et al*., 2009; Levi *et al*., 2016). Pulmonary or lymphatic invasion and metastasis are commonly considered poor prognostic indicators for the management of CMT (Sorenmo *et al*., 2011). Therefore, the effectiveness of selected chemotherapeutic agents in such inherently resistant CMT as well as in recurrent CMT is very limited, most often than not leaving the affected dog with no other therapeutic options.

Some CMT cell lines and tissues express drug resistance genes (drug efflux pumps) inherently, which render the cells resistant to chemotherapeutic drugs even before exposure (Honscha *et al*., 2009; Król *et al*., 2014). *MRP1* and *BCRP* expression was reported in 100% of 103 CMT tissues examined by reverse transcription–PCR (RT-PCR), and five other drug resistance genes were expressed in 64–98% of the tissues (Honscha *et al*., 2009). In another similar study, MRP1 and BCRP expression were investigated in CMT tissues of differing malignancy by immunohistochemistry (Levi *et al*., 2016): MRP1 and BCRP expression was highest in malignant CMT tissues without prior exposure to chemotherapeutic agents. Another study in dogs with CMT reported a strong association between the immunohistochemical expression of MRP1 (in 37.2% of the dogs) with higher histological grade and decreased overall survival (Salgado *et al*., 2015). The study suggested *MRP1* expression as a prognostic indicator independent of lymph node metastasis in dogs with CMT.

Cancer stem cells (CSCs) are a small group of cells (0.1–4%) in the general cancer cell population that can initiate tumours and cause metastasis and recurrence even after apparently successful treatment (Louie *et al*., 2010; Bao *et al*., 2013; Ouzounova *et al*., 2013). CSCs can achieve the said characteristics as a result of their ability to self-renew, live for a long time, resist apoptosis, undergo a dormant state and differentiate into tumour cells (Cocola *et al*., 2009; Bao *et al*., 2013). CSCs are also known to express the drug efflux pumps called ATP-binding cassettes (ABCs), which are responsible for removing anti-cancer agents that enter the cell, effectively rendering CSCs resistant to conventional chemotherapy (Kim *et al*., 2013; Khammanivong *et al*., 2016; Rybicka & Król, 2016).

Studies on cancer have linked tumour initiation, progression, drug resistance in cancer as well as the maintenance of the CSC phenotype to microRNA (miRNA) activity. miRNAs are short (20–24 nucleotides) non-coding RNAs that have regulatory roles in gene expression in multicellular organisms, including mammals (Jansson & Lund, 2012). Up to 1881 precursor and 2588 mature miRNAs have been identified in humans, most of which are conserved among vertebrates and invertebrates (Griffiths-Jones, 2010; Acunzo *et al*., 2015). Predictively, miRNAs regulate up to 60% of all mRNAs, thus they are considered the most ubiquitous mode of post-transcriptional gene regulation (Bartel, 2009). Dysregulated miRNA transcription drives oncogenesis, and many oncomiRs (miRNAs that enhance tumorigenesis) and tumour-suppressor miRNAs have been identified in cancer. Modulating miRNA transcription has already shown promise in revolutionizing anti-cancer therapy. Despite the many studies focusing on miRNAs, their roles in CMT initiation, progression, biology, drug resistance and metastasis have not been fully explored. To date, only one study has described miRNA transcription in CMT stem-like cells, where nine miRNAs were identified as upregulated and 24 miRNAs as downregulated (Rybicka *et al*., 2015). The dysregulated miRNAs target mRNAs such as *TGFBR*, *PDGFRA*, *SOS1*, *SMAD2*, *CHUK* and *MEF2*, which are involved in tumour necrosis factor-beta (TNF-β) signalling, indicating a possible significant role in CMT CSC-like biology.

In the dog with drug-resistant CMT, or with recurrent CMT, which genes are responsible for acquired resistance in the cells and that can be explored and therapeutically targeted? Which signalling pathways are involved in drug resistance, especially in the CSCs of CMT? Which mRNAs and dysregulated miRNAs in the CSCs could be targeted for innovative/novel therapies of CMT? To answer these questions, the following objectives were generated.

The objectives of this thesis were to: 1) develop and characterize a doxorubicin-resistant canine mammary gland adenocarcinoma cell line *in vitro*; 2) evaluate the tumourigenic and metastatic capacities of the induced cell line and the parental cell line *in vivo* in BALB/c and NOD/SCID (non-obese diabetic/severe combined immunodeficient) mouse models; (3) isolate and characterize cells with CSC properties ($CD44^{+}$, $CD24^{-/low}$, aldehyde dehydrogenase $[ALDH]^{+}$) from CMT cells with different doxorubicin-resistant properties; (4) determine the mRNA and signalling pathways activated in the above putative CSCs ($CD44^{+}$, $CD24^{-/low}$, $ALDH^{+}$); and 5) determine the miRNAs and their downstream target genes, and the signalling pathways in the putative CSCs ($CD44^{+}$, $CD24^{-/low}$, $ALDH^{+}$).

The hypotheses are: (1) the expression of one or more of the known drug efflux pumps confers the acquired drug resistance in the CMT cells; (2) the CMT cell lines are tumourigenic in mice; (3) the genes differentially expressed between the drug-resistant cells and parental cells can be potential markers for doxorubicin resistance in dogs undergoing doxorubicin therapies; (4) CSCs can be isolated from the original and doxorubicin-resistant cell lines, but the doxorubicin-resistant cells should have more enriched CSC properties; (5) specific mRNA and signalling pathways will be involved

in enhancing the doxorubicin-resistant and CSC properties; (6) miRNAs regulating specific genes and signalling pathways will be involved in enhancing the doxorubicin-resistant and CSC properties.

In Chapter 3, the development of the doxorubicin-resistant CMT-Star cell line from CMT-Stylo cells is described. Previous work has shown that the CMT tissue from which CMT-Stylo cells are developed show vimentin and proliferating cell nuclear antigen (PCNA) in 44% and 66% of the cells, respectively (Figure 2.1). The CMT-Star cell line was developed by exposing CMT-Stylo cells to increasing concentrations of doxorubicin for an extended duration. The resulting CMT-Star cells were characterized as more resistant to other chemotherapeutic agents. The mRNAs responsible for the acquired resistance in the cells were identified and quantified. In Chapter 4, the tumorigenic and metastatic capacity of CMT-Stylo cells and the doxorubicin-resistant CMT-Star cells were evaluated in mice *in vivo*. In Chapter 5, CSCs were isolated and identified from the two cell lines, and gene expression microarray (44,000 mRNA probes) was used to determine the differentially expressed mRNAs and shed light on dysregulated signalling pathways. In Chapter 6, the miRNA profiles of the CSCs of the CMT-Stylo and CMT-Star cells were determined using a custom-designed canine miRNA microarray (60,000 miRNA probes). The dysregulated signalling pathways were identified, and the specific molecular and biological properties of the miRNAs in the profiles were explored.

The work done in this thesis has developed a drug-resistant CMT cell line and identified genes that are responsible for the development of drug resistance in CMT following exposure to chemotherapeutic agents. These genes could be targeted with inhibitors, in conjunction with chemotherapeutic agents, to enhance their cytotoxic effects on the CMT cells. This work has also isolated and identified CSCs using established CSC markers, successfully profiled the mRNAs and identified the possible associated signalling pathways. This would contribute greatly in guiding further research to identify the specific roles and functions of the dysregulated genes, especially in the CSCs, and for identifying the molecular markers and potential therapeutic targets in dogs with drug-resistant or recurrent CMT. Most importantly, this study has for the first time identified differentially transcribed miRNAs in CSCs as well as drug-resistant CMT. These findings can shed more light on how miRNAs are dysregulated in CMT and CSCs to maintain the stem cell phenotype and enhance cancer cell survival and progression. It has also identified miRNAs that could be further investigated to establish their potentiality as therapeutic targets in CSCs and in CMT with a drug-resistant phenotype. The overall findings in this thesis will contribute to the body of knowledge in the quest for a more effective therapeutic approach for treating dogs with CMT and could possibly be translated to humans with breast cancer.

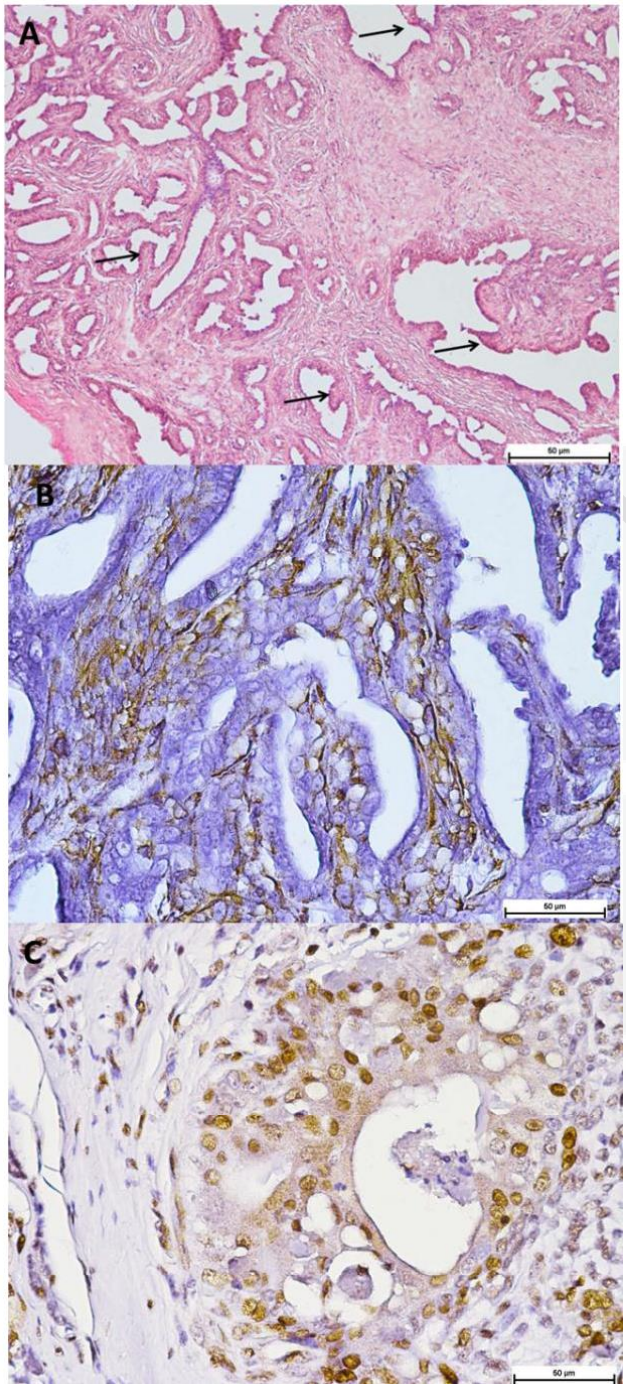

Figure 1.1 : Some characteristics the tumour tissue from which CMT-Stylo cells were developed

H and E staining of mastectomy tissue diagnosed as a tubulopapillary carcinoma, with arrows pointing at papillary structures projecting into the lumen of the tubules (A), vimentin (44%) (B) and PCNA (66%) (C) immunoexpression in the tissue used to develop the CMT-Stylo cells. Browning of the cell membrane or nucleus indicates positive expression of the proteins in the cells. (Bar = 50 µm).

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