เนื้องอกติดต่อระบบสืบพันธุ์สุนัข: การแสดงออกของยีนและโปรตีนที่เกี่ยวข้องกับการดื้อยาระหว่างการได้รับยาวินคริสทีนซัลเฟต

นายพรรษวุฒิ สุดใจดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ภาควิชาสูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

CANINE TRANSMISSIBLE VENEREAL TUMOR: MULTIDRUG RESISTANCE ASSOCIATED GENE AND PROTEIN EXPRESSIONS DURING VINCRISTINE SULFATE ADMINISTRATION

Mr. Pansawut Sudjaidee

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2014

Thesis Title	CANINE TRANSMISSIBLE VENEREAL TUMOR: MULTIDRUG RESISTANCE ASSOCIATED GENE AND PROTEIN EXPRESSIONS DURING VINCRISTINE SULFATE ADMINISTRATION
By	Mr. Pansawut Sudjaidee
Field of Study	Theriogenology
Thesis Advisor	Associate ProfessorKaywalee Chatdarong, D.V.M., M.Sc., Ph.D.
Thesis Co-Advisor	Suppawiwat Ponglowhapan, D.V.M., M.Sc., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Veterinary Science
(ProfessorRoongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate ProfessorPadet Tummaruk, D.V.M., M.Sc., Ph.D.)

.....Thesis Advisor
(Associate ProfessorKaywalee Chatdarong, D.V.M., M.Sc., Ph.D.)

.....Thesis Co-Advisor
(Suppawiwat Ponglowhapan, D.V.M., M.Sc., Ph.D.)

.....Examiner
(Assistant ProfessorSomporn Techangamsuwan, D.V.M., M.Sc., Ph.D.)

.....Examiner
(Associate ProfessorChainarong Lohachit, D.V.M., Dr.Med.Vet)

.....External Examiner
(Assistant ProfessorSukanya Manee-in, D.V.M., M.Sc., Ph.D.)

พรรษวุฒิ สุดใจดี : เนื้องอกติดต่อระบบสืบพันธุ์สุนัข: การแสดงออกของยีนและโปรตีนที่เกี่ยวข้องกับการดื้อยาระหว่างการได้รับยาวินคริสทีนซัลเฟต (CANINE TRANSMISSIBLE VENEREAL TUMOR: MULTIDRUG RESISTANCE ASSOCIATED GENE AND PROTEIN EXPRESSIONS DURING VINCRISTINE SULFATE ADMINISTRATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร.เกวลี ฉัตรดรงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. น.สพ. ดร.ศุภวิวัธน์ พงษ์เลาหพันธุ์, 92 หน้า.

ดุษฎีนิพนธ์ฉบับนี้มีจุดประสงค์เพื่อ 1) ศึกษาย้อนหลังปัจจัยภายในและปัจจัยภายนอกที่มีผลต่อการหายภายหลังการได้รับยาวินคริสทีนซัลเฟต ซึ่งเป็นยาเคมีบำบัดหลักในการรักษาเนื้องอกติดต่อระบบสืบพันธุ์สุนัข 2) ศึกษากลไกการดื้อยาผ่านโปรตีนขนส่งยา (พีไกลโคโปรตีน, โปรตีนเอ็มอาร์พี1 และโปรตีนเอ็มอาร์พี2) และ 3) ประเมินผลทางคลินิกของการใช้ยาแอลแอสพาราจีเนสควบคู่กับยาวินคริสทีนซัลเฟตในสุนัขที่เนื้องอกทนต่อการรักษาด้วยยาวินคริสทีนมาก่อน ปัจจัยที่นำมาศึกษา นำมาจากสุนัขป่วยที่เป็นเนื้องอกติดต่อระบบสืบพันธุ์จำนวน 157 ตัว แบ่งออกเป็น 3 ส่วนคือ ข้อมูลทั่วไปจากตัวสัตว์ ข้อมูลจำเพาะของเนื้องอก และข้อมูลที่เกี่ยวข้องกับการรักษา สุนัขเพศผู้ 75 ตัว และเพศเมีย 82 ตัว อายุเฉลี่ย 6.4±3.61 ปี ป่วยเป็นเนื้องอกชนิดนี้ที่บริเวณอวัยวะเพศหรือนอกตำแหน่งอวัยวะเพศที่มีขนาดแตกต่างกันและได้รับยาวินคริสทีนจำนวนเฉลี่ย 5.0±2.6 เข็ม ระหว่างฤดูกาลที่ต่างกัน นำมาวิเคราะห์การหายแบบสมบูรณ์หรือการหายเพียงบางส่วน พบว่า อายุ เพศและพันธุ์ไม่ส่งผลต่อการรักษาหรือจำนวนเข็มของยาวินคริสทีนซัลเฟต เนื้องอกที่พบในตำแหน่งระบบสืบพันธุ์มีแนวโน้มที่หายสมบูรณ์มากกว่าตำแหน่งอื่น (P=0.08) และเนื้องอกที่มีขนาดเส้นผ่านศูนย์กลางมากกว่า 6 เซนติเมตร ใช้จำนวนครั้งของยาวินคริสทีนซัลเฟตมากกว่าเนื้องอกที่มีเส้นผ่านศูนย์กลางน้อยกว่า 2 เซนติเมตร (P=0.05) ยิ่งไปกว่านั้นมีแนวโน้มที่จะเกิดการหายแบบสมบูรณ์ในภาวะอุณหภูมิเฉลี่ยตลอด 60 วันของการรักษาต่ำกว่า และมีแนวโน้มเกิดการหายแบบไม่สมบูรณ์ในภาวะอุณหภูมิเฉลี่ยตลอด 60 วันสูงขึ้น (P=0.08 และ P=0.07 ตามลำดับ) ผลการศึกษาครั้งนี้อาจนำไปสู่การพยากรณ์ผลของการรักษาด้วยยาวินคริสทีนซัลเฟต ซึ่งปัจจัยที่มีผลต่อการรักษาคือตำแหน่งที่เกิดเนื้องอก ขนาดของเนื้องอกและสภาวะอากาศ การศึกษาในส่วนที่สอง กระทำในสุนัข 2 กลุ่มคือ สุนัขกลุ่มที่ 1 ที่ไม่เคยได้รับการรักษามาก่อน จำนวน 12 ตัว เพื่อเก็บตัวอย่างชิ้นเนื้อระหว่างการรักษาและ สุนัขกลุ่มที่ 2 จำนวน 4 ตัวที่เกิดการดื้อยาขึ้นแล้ว ตัวอย่างชิ้นเนื้อนำไป (i) ตรวจวัดระดับการแสดงออกของเมสเซนเจอร์อาร์เอ็นเอของ เอ็มดีอาร์1 (*MDR1*) เอ็มอาร์พี 1 และ 2 (*MRP1* และ *MRP2*) (ii) ตรวจวัดการแสดงออกของพีไกลโคโปรตีน (P-glycoprotein) เอ็มอาร์พี1 (MRP1) และเอ็มอาร์พี2(MRP2) ด้วยวิธีการย้อมทางอิมมูนโนฮีสโตเคมี และ (iii) ตรวจวัดระดับการแสดงออกของโปรตีนทั้ง 3 ชนิดด้วยวิธีการเวสเทิร์นบลอท จากการศึกษาพบการแสดงออกของยีนและโปรตีนที่เกี่ยวข้องกับการดื้อยาทั้ง 3 ชนิดในทุกตัวอย่าง ในระหว่างการรักษา ไม่พบความแตกต่างระดับเอ็มอาร์เอ็นเอของโปรตีนทั้ง 3 ชนิด พบการแสดงออกของพีไกลโคโปรตีนลดลงในสัปดาห์ที่ 3 เปรียบเทียบกับก่อนการรักษาจากวิธีอิมมูโนฮีสโตเคมี ในกลุ่มที่มีการดื้อยา การแสดงออกของเอ็มอาร์เอ็นเอของเอ็มดีอาร์ 1 ลดลงเมื่อเทียบกับกลุ่มก่อนการรักษา สอดคล้องกับการแสดงออกของพีไกลโคโปรตีนที่ลดลงในกลุ่มที่มีการดื้อยา ขณะที่การแสดงออกของโปรตีนเอ็มอาร์พี 2 เพิ่มขึ้นจากวิธีการอิมมูนโนฮีสโตเคมีในรายที่มีการดื้อยา แต่ไม่พบความแตกต่างของการแสดงออกของโปรตีนเอ็มอาร์พี 1 ระหว่างทั้ง 2 กลุ่ม จากผลของการศึกษาสรุปได้ว่าโปรตีนเอ็มอาร์พี 2 อาจเกี่ยวข้องกับการดื้อยาวินคริสทีนซัลเฟตในเนื้องอกติดต่อระบบสืบพันธุ์สุนัข ควรหลีกเลี่ยงการใช้ยาที่ถูกขับออกด้วยโปรตีนเอ็มอาร์พี 2 ในเนื้องอกติดต่อระบบสืบพันธุ์สุนัขที่มีการดื้อยาเกิดขึ้นแล้ว การศึกษาส่วนที่สามมีจุดประสงค์เพื่อนำยาแอลแอสพาราจีเนสควบคู่กับวินคริสทีนในการรักษาเนื้องอกติดต่อระบบสืบพันธุ์ในสุนัขที่ดื้อยา สุนัขดื้อยาจำนวน 3 ตัวเคยได้รับยาวินคริสทีนมาก่อน หลังเสร็จสิ้นการรักษาพบเนื้องอกดังกล่าว จึงมีการนำยาแอลแอสพาราจีเนสขนาด 10,000 หน่วยสากลต่อตารางเมตรพื้นที่ร่างกายร่วมกับยาวินคริสทีน ภายหลังการรักษาพบว่าสุนัขป่วยทุกตัวหายจากเนื้องอกดังกล่าวและไม่พบการกลับมาเกิดโรคใหม่หลังการรักษา 6 เดือน จากผลของการศึกษาสรุปได้ว่า การให้ยาแอลแอสพาราจีเนสร่วมกับวินคริสทีนทุก 2 สัปดาห์เป็นเวลา 4 ครั้งเป็นวิธีการรักษาที่ให้ผลดีและลดผลข้างเคียงจากการใช้ยาร่วมกันได้เมื่อเปรียบเทียบกับการให้ยาเคมีบำบัดชนิดอื่นก่อนหน้า

โดยสรุปแล้ว ขนาดและตำแหน่งของเนื้องอกและสภาะอากาศระหว่างการรักษามีผลต่อการหายของเนื้องอกติดต่อระบบสืบพันธุ์สุนัข โปรตีนเอ็มอาร์พี 2 อาจเกี่ยวข้องกับกลไกดื้อยาในเนื้องอกชนิดนี้และการใช้ยาแอลแอสพาราจีเนสร่วมกับวินคริสทีนเป็นอีกแนวทางเลือกหนึ่งในการรักษาเนื้อง อกติดต่อระบบสืบพันธุ์สุนัขที่เกิดการดื้อยาขึ้นได้

ภาควิชา	สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์	ลายมือชื่อนิสิต	.....
สาขาวิชา	วิทยาการสืบพันธุ์สัตว์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2557	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

5275967931 : MAJOR THERIOGENOLOGY
KEYWORDS: CANINE TRANSMISSIBLE VENEREAL TUMOR, TREATMENT OUTCOME, MULTIDRUG RESISTANCE, GENE AND PROTEIN EXPRESSION, VINCRISTINE TREATMENT

PANSAWUT SUDJAIDEE: CANINE TRANSMISSIBLE VENEREAL TUMOR: MULTIDRUG RESISTANCE ASSOCIATED GENE AND PROTEIN EXPRESSIONS DURING VINCRISTINE SULFATE ADMINISTRATION. ADVISOR: ASSOC. PROF.KAYWALEE CHATDARONG, D.V.M., M.Sc., Ph.D., CO-ADVISOR: SUPPAWIWAT PONGLOWHAPAN, D.V.M., M.Sc., Ph.D., 92 pp.

The thesis was aimed to: 1) retrospectively find internal and external factors affecting treatment outcome after administrating vincristine sulfate which is the chemotherapeutic drug of choice for canine transmissible venereal tumor (cTVT), 2) study mechanism of drug resistance via ABC-transporter (p-glycoprotein (P-gp), multidrug resistance protein-1 (MRP1) and multidrug resistance protein-2 (MRP2), and 3) clinically evaluate the use of l-asparaginase as a combined drug with vincristine sulfate in animals tolerated to previous vincristine administration. Factors were retrieved from medical records of 157 dogs infected with cTVT and divided into three categories; host general background, tumor details, and treatment details. The male and female dogs (75 and 82 dogs, respectively) aged (6.4±3.61 years) affected with genital and extra-genital cTVT tumor masses of various diameters, and treated with vincristine (5.0±2.6 shots) during various seasons were analyzed for tumor mass complete remission (CR) or partial remission (PR). The age, gender and breed had no effect on treatment outcome or number of vincristine shots. The tumors at the genital area were prone to CR than the others (P = 0.08). The tumors of diameter >6 cm required higher number of vincristine shots than that of diameter <2 cm (P = 0.05). Moreover, there was a tendency that CR was observed with lower average daily ambient temperature at 60 days after enrolled (Temp60) and lower maximal average daily ambient temperature (MaxTemp60) than the PR cases (P = 0.08 and P = 0.07, respectively). The results contributed to the prognosis of the treatment with vincristine sulfate particularly when the treatment is influenced by tumor site, tumor size, and climate. The second study was performed in 12 cTVT affected dogs during vincristine treatments and 4 dogs resistant to vincristine therapy. Tumor samples were submitted to mRNA quantification via quantitative realtime polymerase chain reaction (qPCR), immunolocalization by immunohistochemistry (IHC) and protein expression level analysis by western blot analysis (WB). The *MDR1*, *MRP1* and *MRP2* mRNAs and proteins were detected in all samples. During vincristine treatments, there was no significant difference in all mRNA expressions. The lower expression of P-gp was observed at week3 after the start of treatment whereas MRP1 and MRP2 proteins were not different. In comparison with the untreated group, the resistance group demonstrated lower *MDR1* mRNA expression leading to the lower confirmed by IHC and WB. MRP2 protein (IHC) was significant higher in the resistant group but MRP1 protein expression was no significant difference between the two groups. The results suggested that MRP2 likely involves multidrug resistance mechanism of cTVT. Drugs transported through MRP2-related mechanism should be avoided in the cTVT resistance cases. The third part of this study was aimed to combine l-asparaginase with the standard treatment in resistance cTVT. The resistance cases were composed of three cases that had been treated with vincristine and did not achieve a complete remission. L-asparaginase at 10,000IU/m^2 BSA was combined with vincristine injections. All cases were achieved complete remission and the recurrence was not observed six months after complete treatment. This finding suggested that L-asparaginase in combination with vincristine at every 2 weeks provided a promising result in the resistance cTVT with minimal side effect when compared with the other anticancer drugs suggested previously.

In conclusion, tumor diameter, tumor location and daily ambient temperature during treatment might influence the treatment outcome in cTVT. The MRP2 might be involved in the vincristine resistance mechanism in cTVT and the combination of L-asparaginase with vincristine is an alternative treatment for vincristine-resistance cTVT.

Department: Obstetrics Gynaecology and Reproduction
Field of Study: Theriogenology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

This study was carried out at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The study was financially supported by the Faculty Development Scholarship from Commission on Higher Education, the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and Research Unit of Small Animal Reproduction for facility providing.

First of all, I would like to express my gratitude to my main advisor, Assoc. Prof. Dr. Kaywalee Chatdarong for her valuable guidance in PhD living, giving me the great opportunity knowing the process of scientific learning and understanding research working. I am really appreciated for motivation during my PhD to gain the achievement. I would like to thank Dr. Suppawiwat Ponglowhapan, my co-advisor, for helping and managing during my PhD life. I am really grateful for Assist. Prof. Dr. Sayamon Srisuwattanasagul for giving me a very good advice not only a technique in immunohistochemistry study but also a good take care in my PhD living. I wish to thank Dr. Theerawat Sawangchan-uthai for giving me a valuable advice in qPCR and western blot study and supporting me against the problem in research working. I would like to thank Assoc. Prof. Dr. Padet Tummaruk for a good statistical analysis advice. I also thank Dr. Pichanun Linharattanaruksa for guidance in RNA extraction process.

I would like thank Assoc. Prof. Dr. Meena Sarikaputi and Ms. Suchin Sirisawat at Biochemistry unit, Department of Physiology for an advice in protein separation. A lot of thanks to Veterinarians and OGR staffs at OGR clinic, Small animal teaching hospital of Chulalongkorn University, Dr. Sirichai Techarungchaikul, Dr. Seth Chaovarattana, Dr. Thitida Pakdeesaneha for kind assistances all sample collection. The special thanks for all dogs and owners providing the samples and collaborating in this study. Without you, I would not finish this study. I also thanks to Dr. Paweena Thuwanut, Dr. Nicole Sirisopis Mehl, Dr. Chomanart Thongkittidilok, Dr. Nawarus Prapaiwan and all OGR students for helping me in research working. I also thanks my very best friend ever, Dr. Passorn Sang-urai and Dr. Kamonwatt Sutthisak. Without the support from both of you, I could not pass the difficulties.

Finally, I would like to thank the most important person in my life, mom and dad, for your unconditional love that never ending. I could never come this far without your love. Thanks to my little brother, Nattawut, that never leaves me in every moment. Thanks to my big family, my aunts and uncles, you always walk by my side. Lastly, thanks to you, Sethanun, still are next to me. Without your support, this accomplishment would not been happened.

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Canine transmissible venereal tumor (cTVT) is an important neoplastic disease in the reproductive tract transmitted by cellular transplantation through mucosal tears during natural mating or through nasal mucosa during social behavioral expression. The tumor only occurs in dogs and closely canid species (Murgia et al., 2006). Free-roaming dogs are the important source of disease distribution because of free-range living and poor breeding control. Cytological method is widely used to diagnose TVT because it is simple to perform and causes less pain. Canine Transmissible Venereal Tumor is classified as genital TVT (gTVT) when the tumor masses are located in the genital area and extra-genital TVT (ETVT) when the tumor masses are observed outside genital area (Rogers, 1997). Treatments of choice for cTVT are chemotherapy and radiation therapy (Rogers et al., 1998). The chemotherapeutic agent commonly used is vincristine sulfate, a plant alkaloid, yielding the high response rate but non-responsive cases have been reported (Boscos and Ververidis, 2004; Said et al., 2009; Kunakornsawat et al., 2010). The previous studies suggested that treatment outcome may be related to many factors such as type of cTVT (Santos de Amaral et al., 2007), size of the tumor, age, seasonal effect (Scarpelli et al., 2010) and duration of tumor progression (Boscos and Ververidis, 2004). Moreover, treatment response to cTVT is suggested to be related to P-glycoprotein (P-gp) expression (Gaspar et al., 2010).

Multidrug resistance mechanism (MDR) in neoplastic diseases has been discovered for decades. It causes interruption in cancer chemotherapeutic treatment. Recently, chemotherapy is the treatment of choice for cancer disease but drug resistance is also evident. During treatments, cancer cells are killed by cytotoxic property of chemotherapy although some are not. The

survived cancer cells then generate certain specific structures that compromise cytotoxic property of chemotherapeutic drug and the resistance occurs. The resistance mechanisms are associated with an alteration of cellular proteins that cause a decrease in cytotoxic drug uptake, an increase in drug metabolism or an increase in drug efflux (Gottesman et al., 2002). Proteins on tumor cell surface play an important role in these mechanisms. P-glycoprotein (P-gp), ATP-dependent Binding Cassette (ABC) transporter, is an important membrane integral protein functions as an efflux pump on cell membrane that transports lipophilic agent out of cells for example, vinca alkaloids and anthracyclines. The protein can be seen not only throughout the body such as blood-brain barrier, liver and kidney but also on tumor cell surface (Yamaguchi et al., 2006). P-glycoprotein expression is found in many types of tumor such as hepatoma, colorectal carcinoma, hemangiopericytoma and lymphoma (Ginn, 1996). Studies revealed that the resistance developed *in vitro* cause a reduction in intracellular drug concentration (Uozurmi et al., 2005; Yamaguchi et al., 2006; Yang et al., 2006). In addition to P-gp, multidrug resistance associated proteins (MRPs) are members of the ABC transporter family. These proteins react with negative - charge drugs and drugs modified by glutathione, conjugation, glucosylation, sulfation, and glucuronylation (Gottesman et al., 2002). Interestingly, a correlation between the expression of MRPs and treatment response has been reported (Lee et al., 2007; Noma et al., 2008; Honscha et al., 2009). There were the studies on the expression of P-gp and MRP in paraffin-embedded tissues of cTVT (Gaspar et al., 2010; Gerardi et al., 2014); however, there is no study describing in the expression of mRNA and protein during treatments. The different level of mRNA and proteins expressions may help explain the underlying process related to drug resistance mechanism of cTVT. Better understanding in the mechanism of vincristine resistance cTVT in association with specific proteins, i.e. P-gp or MRPs is beneficial for the development of a novel treatment in dogs developing the drug resistance.

1.2 Literature review

1.2.1 General background

Canine transmissible venereal tumor (cTVT) is a contagious, sexually transmitted tumor commonly occurs in veterinary practice. This tumor is well known in many names such as contagious lymphoma, infectious sarcoma, contagious venereal tumor, venereal granuloma, canine condyloma, transmissible venereal sarcoma or Sticker's sarcoma. Dogs living in areas with high frequency of free-roaming dogs and poor breeding control are at high risk (Rogers, 1997). In 1876, Novinski succeeded to develop cTVT mass by rubbing the excised mass and implanted on scarified genital mucosa. The tumor is transmitted by two different ways. The first is by invading through genital mucosal tears that occur during natural mating or through nasal mucosa during social behavioral expression such as sniffing and licking the genital area of infected dogs. The second is by injecting into subcutaneous areas. The second is used for disease model experiment or maintaining the tumor cell line in experimental study. The mechanism of infection is the transplantation of viable cells through mucosa. In experimental study, viable cells inoculation can induce tumor progression but dead cells cannot. Canine TVT can be transplanted in closely canid species such as coyotes, red foxes, wolves and jackals (Murgia et al., 2006). This tumor is worldwide distributed but the prevalence appears to be higher in tropical and subtropical urban areas. The prevalence previously reported slightly differed among studies depending on the geography for example 11% in Kenya, 32% in Sri Lanka, 10% in Maryland (USA) and 23.5 to 28.6% in India (Das and Das, 2000). Disease occurrence may not differ among genders (Rogers, 1997). The affected dogs are likely to be mature dogs with an average age of 4 to 5 years and being of large breed with weight between 18 and 20 kg (Rogers, 1997).

1.2.2 Clinical signs

External genitalia are the most common affected sites of cTVT. Tumor may present with bloody vaginal discharge, genital area swelling or

malformation, excessive licking or visible mass. Tumor ulceration and necrosis by bacterial infection may cause an abnormal odor. Canine TVT may present in solitary or multiple mass that shows cauliflower-like, nodular, papillary or multilobulated. Regarding ETVT, clinical signs include nasal discharge, sneezing, regional or distance lymph node enlargement, cutaneous swelling or facial deformation (Rogers, 1997). Facial deformity occurs by tumor invasion in orbit or nasal cavity.

1.2.3 Diagnosis

Typical cytology is a useful and effective method to diagnose cTVT due to an exfoliated property of the round cell tumor yields more abundant diagnostic cells. Tumor cells show specific characteristics including dense chromatin, one or two prominent nucleoli and pale blue cytoplasm with the presence of abundant vacuoles. Tumor cells present in two characteristics as lymphocytoid and plasmacytoid cell-shape. Lymphocytoid cell-shape cTVT are smaller and plasmacytoid cell-shape cTVT are ovoid and eccentric located nucleus. These two types of cell morphology are seen only one type of cell or mixed types (Santos de Amaral et al., 2007). Without specific vacuoles characteristic and particularly if the tumor located in atypical location, this may be confused with other round-cell tumor such as histiocytoma, lymphoma, anaplastic mast cell tumor or amelanotic melanoma (Rogers, 1997).

1.2.4 Tumor biology and behavior

At present, tumor origin is still unclear. It is presumed to be undifferentiated round-cell of reticuloendothelial origin. Recently, the immunohistochemical study suggested that cTVT was originated from histiocytic origin (Mozos et al., 1996). Canine TVT was positive when immunoactivated in lysozyme, alpha-1 antitrypsin and vimentin were detected. These three proteins are positive in histiocytoma but not in lymphoma. Some studies demonstrated that viral particles might be involved but cTVT was seen to be developed by viable cells, not by cell particle, cell-free filtrates, freeze cells or dead cells. If viral infection occurs in host cells, cTVT cells should

produce isoantigenic cell surface like host cells but cTVT do not express them. In chromosomal study, cTVT express stable chromosome at 59 ± 5 without differences between regions of the world while the canine chromosome number is 78. Similar cellular characteristics and karyotype of cTVT from different geographic locations suggest that cTVT develops from a common origin and transmitted through the cellular transplantation (Rogers, 1997).

The presence of tumor location throughout the body is used for tumor-type classification. Genital cTVT locates in genital tract in both sexes and is associated with natural mating or social behavior during mating while extragenital cTVT usually occurs in other organs associated with solely social behavior. In natural setting, cTVT is located and limited in the transplanted area however tumor metastasis occurs in puppies or immunocompromized dogs where the tumor grows vigorously. Tumor may metastasis to inguinal lymph node, external iliac lymph node or cutaneous site. There were reports indicated that cTVT growth was found in tonsils, orbits, brain, adenohypophysis, maxillary bone, kidney and other tissues (Das and Das, 2000). Metastasis occurs by mechanical growth extension of cTVT from external genitalia to cervix, uterus and fallopian tube (Bastan et al., 2008). Experimentally, cTVT can be transplanted by viable cell inoculation and this procedure is used for maintaining the tumor cells in dogs without alteration of cellular chromosome and characteristics. Tumor mass from experimental inoculation progresses continually and the regression of tumor enters spontaneous regression seventeen weeks after inoculation (Hsiao et al., 2002). Although, spontaneous regression is seen in experimentally transplanted cTVT but it is rarely happened in natural occurring cTVT (Hsiao et al., 2008).

The phase of tumor growth is classified into three phases. The first phase is progression phase that tumor grows rapidly. Major histocompatibility complex (MHC) class I molecules are lower expression due to lack of surface β_2 microglobulin of tumor cells. The second phase is steady phase in which tumor dose not progress in size. The last phase is resgression phase. In this phase, MHC class I expression is higher and tumor-infiltrating lymphocytes

present in the tumor. These findings relate to the cytokine interaction in the three phases. During progression phase, tumor cells secrete transforming growth factor-beta (TGF-β1) and this cytokine can inhibit the function of Interferon-gamma (IFN-γ) causing inhibition MHC class I (MHC-I) expression. After entering the regression phase, tumor-infiltrating lymphocytes penetrate into tumor tissues and secrete Interleukin-6 (IL-6). IL-6 functions by inhibiting TGF-β1 and restores the function of IFN-γ resulting in the higher expression of MHC-I that supports the function of CD8+ T lymphocytes to compromise tumor cells and initiate tumor regression (Hsiao et al., 2008).

1.2.5 Treatment

CTVT is a unique tumor that responds to various treatment modalities. Treatment is necessary for management cTVT due to spontaneous regression rarely occurs in natural disease. Tumor response is verified after complete treatment. The response is classified into three groups. The first is complete regression which tumor regresses totally without any presence of remnant or nodule. The second is partial regression classified as more than 50% regression in size but not total regression. Tumor still presents and develops in size. The last group is non-response which tumor shows the regression less than 20% and still increasing in size (Rogers et al., 1998). These criteria are used for evaluation of response and resistance development.

Marginal surgical removal of the mass do not consider as an effective treatment because the recurrence rate has been reported as 68% of local recurrence and 33.4 % of metastatic tumor (Rogers, 1997). It is difficult to use wide margin resection in the area of tumor located. Special concerns for cTVT surgical removal are avoidance to traumatize the urethral orifice and to re-contaminate of viable tumor cells from surgical instrument. There was a successful treatment by cryosurgery in one male dog (Rogers, 1997).

Radiotherapy is an effective treatment for cTVT. From the study of Rogers et al. (1998) using cobalt-60 in three fractions which given one week of total dose of 15 Gys yielded complete remission in one to three treatments.

Radiotherapy yields a good response for cTVT but requires skilled users and expensive equipment that limit this treatment.

Chemotherapy is commonly used for cancer treatment for a long time including treatment of cTVT. There are many chemotherapeutic agents used for cTVT treatment such as cyclophosphamide, methotrexate, vincristine, vinblastine and doxorubicin. Both single and combined usages of chemotherapeutic drugs for treating cTVT have been studied. The most common and most effective drug is vincristine, a vinca alkaloid produced from plant named *Catharanthus roseus* (Madagascar periwinkle). Vincristine acts by binding to tubulin dimer that is necessary for mitotic spindle fiber formation in nucleus and cellular division. When vincistine interfere mitotic spindle fiber formation, the cell division is arrested in metaphase. The dosage of vincristine is 0.025 mg/kg BW or 0.5 mg/m^2 Body Surface Area (BSA) with intravenous route administration weekly interval for 3 to 6 injections (Lorimier and Fan, 2001). This drug is used as a single chemotherapeutic agent for a complete response. There is an attempt to use combination treatment of chemotherapeutic agents for example, cyclophosphamide, methotrexate and vincristine to achieve a good response with minor side effects. Side effects are a major concern in treatment with chemotherapy because drugs are given systemically and drugs affects to all rapidly growth cells both neoplastic cells and normal cells including intestinal cells, hair follicular cells or bone marrow. The adverse effects of vincristine are gastrointestinal upset, e.g. vomit and diarrhea, or myelosuppression, e.g. neutropenia and anemia (Das and Das, 2000). The adverse effects depend on individual sensitivity or drug dosage administration. When the adverse effects occur, drug management consideration is important because the lesser drug is given, the more chance to develop the resistance.

1.2.6 Chemotherapeutic drug resistance: trans-membrane drug efflux pump

Chemotherapy plays an important role in cancer therapy because the chemotherapeutic drug acts by suppression the abnormal growth of cancer cells. While the cancer cells are destroyed, they do develop certain

mechanisms for cellular survival through the treatment called cancer drug resistance. For the last decades, a number of studies have been demonstrated that cancer cells try to survive from destructive property of chemotherapeutic drug by many mechanisms. One important mechanism is lowering the intracellular drug concentration to reduce toxic property of chemotherapeutic drug and this mechanism involves reducing drug uptake and increasing drug excretion by particular proteins generated by tumor cells (Gottesman et al., 2002). There are many proteins that function as drug efflux pump for increasing drug excretion outside the cell. Most of these proteins are trans-membrane proteins called multidrug transporters secreting drugs by using energy dependence. The first member of these proteins discovered is P-glycoprotein (P-gp) which is a product of *MDR1* gene. P-gp is a trans-membrane protein in family of ATP-binding cassette (ABC) found throughout the body such as blood brain barrier, liver and kidney (Gottesman et al., 2002). P-gp, a product of *MDR1* gene encodes 170 kDal proteins, is composed of two ATP-binding cassettes and two trans-membrane regions which each of trans-membrane region composes of six domains. P-gp can detect and bind to a wide variety of hydrophobic natural-product drugs entering cell membrane not only chemotherapeutic drugs such as vinca alkaloid or doxorubicin, but also anti-arrhythmic drug or cholesterol-lowering statins. Binding of this drug causes hydrolysis in the first region of ATP binding site and a conformational change of P-gp. Hydrolysis in the second region of ATP-binding site restores the conformation causing drug releasing to extracellular matrix (Gottesman et al., 2002). After discovery of P-gp, the next protein related to drug resistance is Multidrug resistance associated protein (MRP). Multidrug resistance associated protein, unlike P-gp, MRP is the protein that reacts with negatively charge drug or drug modified by glutathione, conjugation, glucosylation, sulfation and glucuronylation (Gottesman et al., 2002). There are many drug-resistant-related MRPs but the most important MRPs in dogs are MRP1 and MRP2. The expression of P-gp and MRP has been demonstrated in many tumors such as canine transitional cell carcinoma (Lee et al., 2007), mast cell tumor (Nakaichi et al., 2007) and canine transmissible

venereal tumor (Gaspar et al., 2010; Gerardi et al., 2014). More information of the expression of these drug-related resistance proteins may explain the vincristine resistance mechanism in cTVT.

1.3 Objectives of the thesis

1.3.1 To study the current disease status of cTVT and treatment outcome of vincristine sulfate administration

1.3.2 To investigate the mRNA and protein expressions of multidrug resistance gene (*MDR1*) and multidrug resistance associated protein genes (*MRP1* and *MRP2*) in cTVT during vincristine sulfate treatment

1.3.3 To investigate the mRNA and protein expressions of multidrug resistance gene (*MDR1*) and multidrug resistance associated protein genes (*MRP1* and *MRP2*) in resistance cTVT comparing with untreated cTVT

1.3.4 To study the combination of L-asparaginase with vincristine used for treatment of resistance cTVT

1.4 Hypothesis of the study

1.4.1 There are associations of patient's factors or tumor characteristics and cTVT treatment outcome

1.4.2 The mRNA and protein expressions of multidrug resistance gene (*MDR1*) and multidrug resistance associated protein genes (*MRP1* and *MRP2*) are different between before and after treatment with vincristine sulfate

1.4.3 The mRNA and protein expressions of multidrug resistance gene (*MDR1*) and multidrug resistance associated protein genes (*MRP1* and *MRP2*) are different between untreated and resistance cTVT

1.4.4 The combined treatment of L-asparaginase and vincristine could be used for treatment of resistance cTVT

1.5 Keywords: Canine transmissible venereal tumor, Treatment outcome, Multidrug resistance, Gene and protein expression, Vincristine treatment

CHAPTER II

Factor affecting on treatment outcome in canine transmissible venereal tumor

2.1 Abstract

Canine transmissible venereal tumor (TVT) is a contagious neoplastic disease commonly seen in tropical and subtropical area in the countries where the control of strayed dog population is poor. This study aimed to summarize current disease status and evaluate factors affecting treatment outcome to vincristine sulfate which is the chemotherapeutic drug of choice. Studied factors were divided into three categories; general background (i.e. age, gender, breed), specific descriptions (i.e. tumor diameter, tumor site, season of the year), and treatment details (number of vincristine shots, meteorological parameters). One hundred and fifty-seven medical records were included with 100 achieved complete treatments and enrolled in investigation of treatment outcome. Treatment outcome was classified as complete remission (CR) and partial remission (PR) at the week eighth of treatment. Moreover, the corresponding factors affecting number of vincristine shot to commit CR were evaluated. The age, gender and breed had no effect on treatment outcome or number of vincristine shots. The tumors at the genital area were prone to recovered (CR) than the others (P = 0.08). The tumors of diameter >6 cm required higher number of vincristine shots than that of diameter <2 cm (P = 0.05). Moreover, there was a tendency that CR was observed with lower mean temperature at 60 days after enrolled (Temp60) and lower maximal temperature (MaxTemp60) than the PR cases (P = 0.08 and P = 0.07, respectively). This result may contribute to the prognosis of the treatment with vincristine sulfate particularly when the treatment is influenced by tumor site, tumor size and climate.

2.2 Introduction

Canine transmissible venereal tumor (TVT) is an immune-related contagious neoplasm, commonly occurred in the reproductive tract (Das and Das, 2000). The tumor spreads widely in tropical and subtropical urban area where controls of free-roaming dog population are limited (Ganguly et al., 2013). The transmission was through mucosal abrasion during natural coitus or behavioral social expression between dogs, such as sniffing, licking or biting (Otomo et al., 1981). TVT is seen in genital and extra-genital site (Das and Das, 2000). The prevalence has been reported in different geographical areas: 11% of the total number of tumor in canine patients was observed in Kenya, 32% in Sri Lanka, 23.5 – 28.6% in India and 10% in Maryland (USA) (for review: Das and Das, 2000). Chemotherapy, especially vincristine sulfate that given intravenously once a week, is the treatment of choice yielding 90% of complete response after treatment (Ganguly et al., 2013). However, partial response is possibly observed with vary remission rate (Rogers et al., 1998). Treatment outcome is generally individual depending on disease severity, living environment and self-immunity (Das and Das, 2000). The experimental inoculation found that spontaneous regression of TVT was related to interleukin-6 (IL-6) released by tumor-infiltrating lymphocytes which restore host defense mechanism by suppressing TGF-β1 and promoting CD8+ T-lymphocyte to initiate tumor cell destruction (Hsiao et al., 2004). This scheme also presented in natural infected cases treated with vincristine. Thus, host immunity may play an important role in the regression of clinical TVT (Gonzalez et al., 2000).

Scarpelli et al. (2010) revealed TVT cases required more number of vincristine shots to suppress tumor growth during hot and wet season compared to cool and dry season. This might be due to the heat stress resulting in impairment of peripheral natural killer cell function as shown in the pigs (Hicks et al., 1998). The increase in somatic cell count and incidence of clinical mastitis was also presented in dairy cows raised in high ambient temperature (Lacetera et al., 2005). Thus, the host defense immunity is likely

compromised by thermal or heat stress resulting in impaired pathogen clearance.

The aims of this study was to retrospectively find the associations between host details, tumor characteristics, seasons and host responses to vincristine treatments in Bangkok Metropolitan, a city in the tropical area where stray dog population remained a social problem.

2.3 Materials and Methods

2.3.1 Data Source: Medical records of 157 TVT cases of total 48,173 cases visited the Division of Obstetrics, Gynaecology and Reproduction at Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University between March 2007 to March 2012 (60 mths) were included. TVT was diagnosed by cytological method: impression smear or fine needle aspiration and stained with commercial modified Giemsa (Diff-Quick, SE Supply, Bangkok, Thailand). TVT cells revealed round to oval cells, increased nucleus-cytoplasmic ration, and coarse chromatin nucleus with vacuolization cytoplasm which typically found in TVT while tissue biopsy and histopathological method were used for diagnosed extra-genital TVT.

2.3.2 Data category: Data collected from medical records were: 1) general background containing animal age at the day of diagnosed, gender and breed; 2) specific details including tumor diameter (cm), tumor site and season; and 3) treatment details (number of vincristine shots and temperature parameter 60 days during treatment). All cases were treated by vincristine intravenously once a week. Treatment outcome were classified into complete remission (CR) and partial remission (PR) after eight vincristine continuous administrations (complete treatment) (Rogers et al., 1998). Complete remission was defined as total regression of TVT mass while partial remission was classified as tumor

regressed less than 50% compared with tumor size before treatment or tumor mass and cells were found after finished 8 vincristine administration.

Of 157 dogs, 100 had regular visits throughout eight treatments, therefore, were enrolled to test effects of gender, breed, tumor site, season, number of vincristine shots and climate parameters during 60 days of treatment on treatment outcome, whereas only 69 and 51 had records for age and tumor diameter, respectively. To evaluate the influence of age, gender, breed, season and tumor diameter on treatment outcome, the data was classified using the following criteria; age was classified into two groups as less than 5 and more than 5 years old, gender was classified as male and female, breed was classified as pure and mixed breed, tumor diameter was categorized into 4 groups as less than 2 cm, 2 to 4 cm, 4 to 6 cm and more than 6 cm which divided from frequency distribution, tumor site was classified as genital TVT, extra-genital TVT and mixed type, seasons were divided into hot, rainy and cool seasons.

2.3.3 Meteorological data: Meteorological data were collected from Bangkok Metropolitan meteorological station, Thai Meteorological Department, Ministry of Information and Communication Technology, Thailand from January, 2007 to June, 2012. Seasons in Thailand were classified as hot (February 15th to May 14th), rainy (May 15th to October 14th) and cool (October 15th to February14th). Climates in each season were described as mean temperature, mean relative humidity (RH) and mean temperature heat index (THI). The THI was calculated for each day from the formula: THI = DB – [0.55-(0.55 x RH)] x (DB-58) where DB is the average daily temperature and RH is the average daily humidity (Kelly and Bond, 1971). Mean temperatures in hot, rainy and cool season were 29.8±1.71°C (range: 18.8 – 33.7), 29.1±1.16°C (range: 25 – 33.6) and 27.8±1.56 °C (range: 21.4 - 31), respectively. Mean relative humidity was 71.8±6.9% (range: 46 – 92) in hot season, 77.1±6.0% (range: 61 – 94) in rainy season and

67.2±9.24% (range: 46 – 95) in cool season. Mean THI in hot, rainy and cool seasons were 81.2, 81.0 and 77.8.

2.3.4 Temperature classification: The first day dogs enrolled in the treatment program was used to define cases to each season. Comparisons of means temperature (Temp60), 24-h maximum daily temperature (MaxTemp60), and 24-h minimum daily temperature (MinTemp60) during 60 days period after the onset of treatment between CR and PR cases were calculated. Similarly, means daily RH and THI during 60 days periods after the onset of treatment (RH60 and THI60, respectively) of CR and PR cases were compared.

2.3.5 Statistical analysis: Statistical test was carried out by Statistical Analysis System software (SAS version 9.0, Cary, NC, USA). The continuous data were presented as mean ± SD and discrete data were presented as percentage. Chi's square was used to compare percentages of CR within ages, genders, breeds, tumor diameters, tumor sites, seasons and numbers of vincristine shots. General linear model (GLM) was used to analyze effects of climate factors (i.e., Temp, Temp60, Max Temp60, Min Temp60, RH60 and THI60) with treatment outcome. The statistical model included the effects of age, gender, breed, tumor diameter, tumor site, season, and number of vincristine shots. Dependent variable was treatment outcome (%CR). Least-square means were calculated from each class of factors and were compared by using least significant difference test. $P< 0.05$ was regarded to be statistically significant.

2.4 Results

2.4.1 General background: The mean body weight of 157 dogs affected with TVT was 19.6±7.11 kg (ranged 4 – 46.5 kg) with mean age at 6.4±3.61 years (ranged 1 – 21 years). The proportion of male (75/157) and female (82/157) was similar (47.7% and 52.3%, respectively). The dogs were pure breed (24/157) (i.e. 6 bangkaew, 3 golden retriever, 5 labrador retriever and others) and mixed

breed (133/157). All cases were petted outside the house and got infection from free-roaming dogs.

2.4.2 Specific details: TVT was presented mostly in the genital area (135/157, 86%) with minority in the extra-genital area (17/157, 10.8%) and five cases in both area (5/157, 3.2%). Specific sites of TVT mass recorded in 153 of 157 were penile surface (30/153, 19.7%), bulbus glandis (21/153, 13.7%), prepuce (10/153, 6.5%), vagina (52/153, 34%) vulva (23/152, 15%) and other areas (17/148, 11.1%). Bleeding was found in most cases (143/153, 93.2%). Nine extra-genital TVT cases and one case with 0.5-cm diameter located in the vagina without bleeding were presented. Of 157 cases, 81 cases were reported of tumor diameter of 3.8±3.9 centimeters (range: 0.2 – 30 centimeters).

Table 1 Characteristics and clinical findings in 100 cases with transmissible venereal tumor and factors affecting on treatment outcome classified as complete remission (CR) and partial remission (PR)

	CR	PR	*P*-value
General details			
Age (years), N (%)			
≤5 years	29 (76.32)	9 (23.68)	0.49
>5 years	29 (82.86)	6 (17.14)	
BW (kg), mean±SD	18.4±6.58	18.1±7.46	0.86
Gender, N (%)			
male	39 (82.98)	8 (17.02)	0.81
female	43 (81.13)	10 (18.87)	
Breed, N (%)			
pure breed	16 (84.21)	3 (15.78)	0.78
mixed breed	66 (81.48)	15 (18.52)	
Specific details			
Diameter, N (%)			
<2 cm	18 (85.71)	3 (14.29)	0.20
2 - 4 cm	16 (100)	0	
4 - 6 cm	6 (75)	2 (25)	
>6 cm	7 (87.5)	1 (12.5)	
Tumor sites, N (%)			
genital	70 (83.33)	14 (16.67)	0.08
extra-genital	11 (84.62)	2 (15.38)	
mixed	1 (33.33)	2 (66.67)	
Season, N (%)			
hot	27 (77.14)	8 (22.86)	0.13
rainy	26 (76.47)	8 (23.53)	
cool	29 (93.55)	2 (6.45)	
Treatment details			
Dosage (mg/kg BW), mean±SD	0.026±0.003	0.028±0.006	
Shots, N (%)			
≤5 shots	26 (92.86)	2 (7.14)	0.08
>5 shots	56 (77.78)	15 (22.22)	

Different superscript indicates statistically difference of the data in the same column ($P<0.05$)

2.4.3 Treatment outcome: Analysis of treatment outcome was performed in 100 dogs. The rest were excluded due to discontinuation of treatment by some reasons: the dogs were undergone side effects, owner visits were aborted without reasons. Of 100 dogs, 76 cases (76%) were treated with CR and 24 cases (24%) showed PR. Mean numbers of vincristine shot to CR was 5.0±2.6 (median was 5 injections). From this finding, median of this treatment leaded to classify the number of vincristine shot as less than 5 and more than 5 for the analyses. There were no significant differences of treatment outcome (%CR) between/among groups of age, gender, breed, tumor diameter, tumor site, season and number of vincristine shots (Table 1). However, there was a tendency that the dogs with tumor mass at the genital site had higher percentage of CR than those at both sites (P=0.08). Moreover, the dogs received >5 vincristine shots were prone to commit CR than those received ≤5 shots (P=0.08). There were no effects of age, gender, breed, tumor site and season on the number of vincristine shot required to complete remission (Table 2). The dogs with tumor diameter of <2 cm required less number of vincristine shots than those with >6 cm tumor masses (P=0.05) (Table 2). Also, the PR cases were likely observed at higher Temp60 (P=0.08) and MaxTemp60 temperature (P=0.07) than the CR cases (Table 3).

Table 2 Characteristics and clinical findings with transmissible venereal tumor and factors affecting on number of vincristine shots to commit complete remission (76 cases)

	Mean shots	*P*-value
General details		
Age		
≤5 years	7.2±0.32	0.12
>5 years	6.6±0.28	
Gender		
male	6.9±0.32	0.87
female	7.0±0.28	
Breed		
pure breed	6.5±0.55	0.20
mixed breed	7.1±0.40	
Specific details		
Diameter		
<2 cm	6.6±0.43[a]	0.05
2 - 4 cm	7.2±0.45[a,b]	
4 - 6 cm	6.8±0.67[a,b]	
>6 cm	8.2±0.62[b]	
Tumor sites		
genital	6.8±0.26	0.34
extra-genital	7.6±0.52	
mixed	6.5±0.98	
Season		
hot	7.2±0.36	0.37
rainy	7.3±0.37	
cool	6.6±0.36	

Different superscript indicates statistically difference of the data in the same column ($P<0.05$)

Table 3 Response to vincristine treatment according to temperature parameter 60 days during treatment (N=100)

	Response to vincristine treatment		
	CR (N=76)	PR (N=24)	*P*-value
Temp60 (℃)	28.7±0.11	29.2±0.24	0.08
MaxTemp60 (℃)	33.6±0.12	34.1±0.26	0.07
MinTemp60 (℃)	25.3±0.15	25.7±0.31	0.21
RH60 (%)	72.2±0.58	72.9±1.24	0.60
THI60	79.7±0.21	80.5±0.45	0.13

CR = complete remission, PR = partial remission, Temp60 = mean temperature, MaxTemp60 = mean maximum temperature, MinTemp60 = mean minimal temperature, RH60 = mean percentage of relative humidity, THI60 = mean temperature heat index

2.5 Discussion

Factors affecting treatment outcome: The mean age of the dogs in this study was 6.4±3.6 years old which was higher than the previous studies; 3.9 – 4.5 years (Das and Das, 2000). This finding suggested that older dogs could be infected with TVT as possibly as younger dogs although most infected cases usually were in the age of maximum sexual activity (Das and Das, 2000). TVT in this study presented an equal ration between the male and female which was similar to the previous study (Scarpelli et al., 2010). While the female dogs have been reported as more susceptible to the infection than the males (Das and Das, 2000), some studies reported of more cases in the male than female (Osipov and Golubeva, 1976; Brown et al., 1980; Boscos, 1988). Breed susceptibility for TVT infection has not been reported (Ganguly et al., 2013). In this study, most of the infected cases were mixed breed (84.2%) similar to the study by Kunakornsawat et al. (2010) which might result from mixed breed dogs are pet free-roaming that population control is the problem. Moreover, this study demonstrated most of the tumor masses in extra-genital organs (9/10) had no bleeding, indicating that bleeding is not a common sign in the extra-genital TVT cases. Tumor characteristics likely influenced treatment outcome. In this study, extra-genital TVT was prone to PR than the genital TVT

which was similar to the study of Gaspar et al. (2010) that stated extra-genital TVT responded to vincristine treatment less than genital TVT.

Factors affecting number of vincristine shots: In this study, PR was found as 24% which was nearly one-fourth of all cases. Said et al. (2009) showed one case (1/30) refractory to vincristine treatment after six-week administration. Scarpelli et al. (2010) suggested that the older cases used more injections than younger cases. However, in the current study, there was no significant difference between age groups. The other factor affecting number of vincristine injection required to CR was the tumor diameter demonstrated by the more vincristine injections needed to reduce the tumor size of over 6 cm than those less than 2 cm which was similar to the study of Scarpelli et al. (2010) stated that larger tumor required more time to regress completely than a smaller one. Scarpelli et al. (2010) mentioned that the larger tumor contain more tumor cells produced more immunosuppressive factors could retard tumor clearance and require more injections of vincristine treatment.

In this study, there was a tendency that less number of vincristine shots was required in the cool season than the hot season. Moreover, the Temp60 and MaxTemp60 were higher in the PR than CR cases suggesting that high temperature likely tumor reduction via suppression of host immunity system. Similar finding has been reported by Scarpelli et al. (2010) suggesting that TVT treatment by vincristine administration during hot and rainy months was longer and less effective than therapy in cold and dry months. High temperature condition might attribute stress to tumor-bearing dogs which interfere cellular immunity and delay tumor clearance resulting in the more vincristine shots for treatment (Medary et al., 1996; Scarpelli et al., 2010). During high temperature condition, cellular mechanism suppression has been reported in the cows (Lacetera et al., 2005). Hekman et al. (2014) suggested that the chronic stress such as from environmental temperature might induce the release of glucocorticoids which associated with leukopenia, lymphopenia and reduce the leucocyte phagocytic activity. From this finding, in high temperature condition might limit tumor clearance in TVT cases.

For conclusion, understanding factors affecting vincristine therapy might help practitioners to predict the success of treatment outcome. The tumor at extra-genital area and the large tumor size seemed to compromise the treatment outcome of TVT. Moreover, the treatment response in TVT might be associated with host immune response by reducing host immunity, and delaying tumor clearance during the high temperature.

Chapter III

Multidrug resistance gene and protein expression in canine transmissible venereal tumor during vincristine treatment

3.1 Abstract

Multidrug resistance process is an important obstacle in both human and veterinary oncology treatment. Canine transmissible venereal tumor (cTVT) yields a good response with vincristine treatment but the resistance was documented. The resistance mechanism in cTVT is still puzzled. P-glycoprotein (P-gp) and Multidrug resistance associated protein1 (MRP1) were believed that might be involved in multidrug resistance mechanism in cTVT. This study was aimed to determine the *MDR1*, *MRP1* and *MRP2* mRNA and protein expression during vincristine treatment and in resistance cases. Tumor mass was collected from twelve dogs diagnosed as genital cTVT once weekly at the week0, week1, week2 and week3 prior to treatment (TVT1) and the other four cases diagnosed as the resistance cTVT was sampled before treatment (TVT2). The samples were submitted to quantify mRNA expression via quantitative realtime polymerase chain reaction (qPCR) while immunohistochemistry (IHC) was used for immunolocalization and western blot analysis (WB) was used for protein expression level analysis. From this study, *MDR1*, *MRP1* and *MRP2* mRNA was detected in all samples that caused P-gp, MRP1 and MRP2 protein expression in IHC and WB. During vincristine treatment, there was no significant difference in *MDR1*, *MRP1* and *MRP2* mRNA expression. The lower expression of P-gp was observed at week3 after treatment compared with week0 while MRP1 and MRP2 expressed no difference in IHC or WB. In resistance cTVT, *MDR1* mRNA expression was lower in resistance (TVT2) compared with untreated group (TVT1) while *MRP1* and *MRP2* mRNA was not significant difference between resistance and untreated group. P-gp expression was significant lower in the resistance both in IHC and WB while MRP2 was significant higher in the resistance (IHC) but MRP1 expression was no significant difference between these two groups. The results suggested that MRP2 might be involving in multidrug resistance mechanism of cTVT more than P-gp.

3.2 Introduction

Canine transmissible venereal tumor (cTVT) is a contagious tumor commonly seen in tropical or subtropical where population control is poor (Das and Das, 2000). Vincristine is a drug of choice used for treatment yielding 90% effectiveness but the resistance has been documented (Ganguly et al., 2013). CTVT is a unique tumor that progression and regression relates with host immunity. In progression phase, the tumor cells release transforming growth factor-β1 (TGF-β1) suppress interferon-γ (IFN-γ) not to induce major histocompatibility complex class I (MHC class I) expression causing tumor cells invade host immunity detection but in regression phase, tumor-infiltrating lymphocytes localize in tumor mass and release interleukin-6 (IL-6) which inhibit TGF-β1 and induce IFN-γ causing the expression of MHC-class I that CD8+-T-cells could detect and eliminate the tumor cells (Hsiao et al., 2004). Cancer drug resistance is a major obstacle during cancer treatment. The resistance develops and lets cancer cells survive through the treatment. There are three major mechanisms of chemotherapy resistance: first, decrease hydrophilic drug uptake through intracellular drug influx, second, altered intracellular process to neutralize cytotoxic effect and increase energy-dependent transmembrane drug efflux pumps (Hsiao et al., 2004; Szakacs et al., 2006). ATP-binding cassette (ABC) transporters are the most commonly drug efflux pump researched in cancer treatment (Gillet et al., 2007). In human, ABC transporters divided into seven subfamilies (ABCA-ABCG). The well-known member of ABC transporters is P-glycoprotein (P-gp: product of *MDR1* gene) which is grouped in the subfamily ABCB (Honscha et al., 2009). P-gp composes of two transmembrane domains and two nucleotide-binding domains that substrate binds to intracellular binding site and transports to extracellular

space by hydrolysis of ATP (Honscha et al., 2009). P-gp transports wide range of hydrophobic chemotherapeutic drug i.e. anthracyclines, vinca alkaloids (Honscha et al., 2009). Another ABC transporters related in ABCC subfamily are MRP1 and MRP2 (products of *MRP1* and *MRP2* gene, respectively) (Gottesman et al., 2002). These two proteins consist of P-gp like structure with additional transmembrane domain at the N-terminal end (Gillet et al., 2007). MRPs transport broad spectrum of anionic chemotherapeutic drugs or gluthatione conjugates, glucuronides and sulfates i.e. metrotrexate, etoposide, doxorubicin and vincristine (Gillet et al., 2007). There were many studies focused on ABC transporters in both human (Chan et al., 1991; Baldini et al., 1995; Tada et al., 2002; Materna et al., 2004) and veterinary oncology (Ginn, 1996; Mealey et al., 1998; Petterino et al., 2006; Honscha et al., 2009) but there was no study in canine transmissible venereal tumor (cTVT) during vincristine treatment. So, this study was aimed to study mRNA expression of *MDR1*, *MRP1* and *MRP2* and proteins expression of P-gp, MRP1 and MRP2 during vincristine treatment and the resistance in cTVT. From this knowledge may understand and suggest the selection for chemotherapeutics in resistance cTVT administration and management.

3.3 Materials and Methods

3.3.1 Experimental design

The study was composed of two experiments

Experiment I: Development of *MDR1*, *MRP1* and *MRP2* mRNA and protein expression of during vincristine treatment.

Experiment II: *MDR1*, *MRP1* and *MRP2* mRNA and protein expression in the vincristine resistance cases.

3.3.2 Animals

Tumor mass were collected from 16 dogs diagnosed as genital cTVT by cytological methods and stained with commercial modified Giemsa stain (Diff-Quick®, SE Supply, Bangkok, Thailand). All tissue sampling were reviewed under the Chulalongkorn University Animal Care and Use Committee (CU-ACUC)-approved protocol (approval no. 12310011) with owners providing consent form. (12 untreated VS 4 resistance). Twelve cases were assigned standard treatment for cTVT using vincristine intravenously once weekly until complete remission (TVT1) while four cases were treated depending on oncologist decision (TVT2). TVT1 group was anesthetized and biopsy once weekly before treatment for 4 times (week0, week1, week2 and week3) but TVT2 group was sampled before treatment. Tissue biopsy was cut into $1cm^3$ by radiofrequency incision for blood coagulation, washed in isotonic saline solution and then cut into 3 pieces: the first, $0.3cm^3$ for quantitative polymerase chain reaction (qPCR), the second, $0.3cm^3$ for immunohistochemistry (IHC) and the third for western immunoblotting (WB). Tissue samples for qPCR and WB were snapped frozen in liquid nitrogen and kept in -80°C until extraction (Linharattanaruksa et al., 2013b) while those for IHC were immersed in 4% paraformaldehyde in phosphate buffered solution for 48 hrs and then moved and kept in 70% ethyl alcohol until tissue processing (Linharattanaruksa et al., 2013a).

3.3.3 Quantification of mRNA expression

3.3.3.1 RNA extraction

Tissue mass was ground with sterile mortar and pestle. Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) in room temperature following the manufacturer's instructions. The tissue powder was mixed with buffer RLT containing 1% β-mercaptoethanol (β-ME) and

homogenized by vortex for 30 sec. The lysates was centrifuged at full speed for 3 min. The supernatant was transferred to a new sterile microcentrifuge tube and mixed with 70% ethanol in 1:1 ration. 700µl of the solution was transferred to RNeasy spin column and centrifuged at ≥8000 x g for 30 sec. the pass-through solution was discarded and 700µl of buffer RW1 was added and centrifuged at ≥8000 x g for 15 sec. The pass-through was discarded and 500µl of buffer RPE was added and centrifuged at ≥8000 x g for 15 sec. then, the pass-through was discarded and RPE was added and centrifuged at full speed for 2 min. then, the RNA was eluted by nuclease-free water. Total RNA solution was determined the concentration and purity by spectrophotometry at 260 and 280 nm (Nanodrop ND2000, Wilmington, Delaware, USA) which the acceptance ratio of absorbance was in range 1.8-2.1. The RNA integrity was determined by 1% agarose gel electrophoresis with ethidium bromide staining. The presence of 18s and 28s rRNA band was acceptable. The RNA solution was treated for genomic DNA contamination removal using RNA-free DNA treatment kit (Promega, Madison, USA) at 37ºC for 30 min and kept in -80ºC until Reverse transcription was performed.

3.3.3.2 Reverse Transcription (RT)

The complementary DNA (cDNA) was synthesized by Reverse transcription kit (Omniscript First Strand cDNA Synthesis kit; Invitrogen, Calsbad, CA, USA) following the manufacture's instruction. Ten µl of RNA solution was incubated with 2 µl of random primer (100µM), 2 µl of 5 mM deoxynuclrotide triphosphate (dNTP) mixed, 2 µl of 10x Buffer RT, 0.25 µl of RNase inhibitor (40 units/µl), 1 µl of Omnicript RT and 2.75 µl of RNase free water at 37ºC for 60 min. A mastermix of RT reagent was prepared once for variation minimization.

3.3.3.3 Quantitative PCR

The Quantification of each gene expression was performed by ABI7300 realtime PCR system (Applied Biosystem®, Life Technologies, NY, USA). The qPCR master mixes were composed of 10 µl of KAPA SYBR Fast qPCR kits (KAPA Biosystems, MA, USA), 0.4 µl of each forward and reverse primer, 1 µl of cDNA template (20ng) and nuclease-free water. The qPCR condition was run as Taq activation at 95ºC for 5 min and followed by 40 cycles of denaturation at 95ºC 30 sec, appropriate annealing temperature of each gene at 30 sec, extension at 72ºC 20 sec and primer melting temperature process at 78.5ºC 30 sec, then plate read in every cycle. The final extension was done at 72ºC 5min and followed by dissociation curve analysis. The qPCR reaction was run in duplicate. Absolute concentration of PCR product was calculated comparing with the Ct of the standard curve from PCR product serial dilution (Swangchan-Uthai et al., 2011) and expressed as fg/20 ng cDNA (Linharattanaruksa et al., 2013b).

Table 4 Primer sequences for *MDR1*, *MRP1*, *MRP2*, *GAPDH* and *RP5s* with expected product's length and specific annealing temperature used for quantitative real-time PCR

Gene name	Accession number	Primer sequence	Annealing temperature (°C)	Product's length (bp)
MDR1	NM_001003215.1	5'-TTGCTGGTTTTGATGATGGA-3'	55	190
		5'-CTGGACCCTGAATCTTTGG-3'		
MRP1	NM_00100271.1	5'-GAACCATCCATGACCTCAATC-3'	55	200
		5'-GCACACTCCTTCTTCCCAGTTCT-3'		
MRP2	NM_001003081.1	5'-ACAACCTTAGCATAGGGCAGAG-3'	52	193
		5'-ATGATGGTGTGTAGCCTGTGAG-3'		
GAPDH	XM_003434387.2	5'-ATTCCACGGCACAGTCAAG-3'	52	117
		5'-TACTCAGCACCAGCATCACC-3'		
RP5s	XM_533568.4	5'-TCACTGGTGAGAACCCCCT-3'	62	141
		5'-CCTGATTCACACGGCGTAG-3'		

3.3.4 Level of protein expression using Immunohistochemistry

Tissue samples were embedded in paraffin wax, sectioned of 5 μm and placed on slides. The samples were submitted to heamatoxylin and eosin staining for cell morphological study. The cytomorphological study was classified into three types as lymphocytoid; round cells with concentric nucleus, plasamacytoid; oval-shaped cells with eccentric nucleus and mixed type that presented both cell types in equal ration (Santos de Amaral et al., 2007). The immunohistochemical procedure was carried out by avidin-biotin method followed by manufacture's direction (Vectastain® ABC elite kit, Vector Laboratories, CA, USA). Briefly, tissue sections were deparaffinized and rehydrated with graded alcohol. Heat-antigen retrieval was performed by microwave oven for P-gp and MRP1 while Autoclave machine was used for MRP2. Tissue sections were immersed in 0.01M citric acid buffer (pH6.0). Endogenous peroxidase activity was blocked in 3% hydrogen peroxide in absolute methanol for 10 min in room temperature. Sections were washed by Phosphate buffer saline (PBS) and incubated with normal horse serum in humidified chamber 30 min at room temperature for non-specific binding prevention. Tissue sections were incubated with primary monoclonal antibody in humidified chamber at 4°C overnight (Monoclonal Antibody clones: 1:50 C494 for P-gp (Petterino et al., 2006): catalogue number 517312, EMD Milipore corporation, CA, USA, 1:50 MRPm6 for MRP1 (Tada et al., 2002): catalogue number ab3371, Abcam CL, Cambridge, UK and 1:50 M_2III-6 for MRP2 (Tada et al., 2002): catalogue number ab3373, Abcam CL, Cambridge, UK; presented in Table 1). The sections were rinsed and incubated with the bionylated anti-mouse made from horse antibody (Vector Laboratories, CA, USA) for 30 min. The sections were incubated with DAB peroxidase substrate (DAB peroxidase substrate kit, Vector Laboratories, CA, USA), counterstained with Mayer's

hematoxylin and then mounted with glycerine-gelatin. Canine liver sections were used as positive control for P-gp, MRP1 and MRP2 detection. Each section was evaluated using computing image analysis program (ImagePro PLUS version6.0). Ten microscopic areas which corresponded to 0.0845 mm^2 of real tissue area with 400X magnification were randomly chosen from each field. Expression evaluation via expression index which derived by a percentage expression and intensity score (Expression index=[%expression x intensity score]/100). The intensity was graded as 0=negative staining, 1=weak staining, 2=moderate staining and 3=strong staining which graded by image analysis program (Linharattanaruksa et al., 2013a).

3.3.5 Levels of protein expression using western blot analysis

3.3.5.1 Tissue lysate preparation and protein extraction

Frozen tissue mass was ground into powder with a sterile mortar and pestle surrounded in liquid nitrogen. Tissue powder was put into 2 ml microcentrifuge tube and added with lysis buffer (500 µl). The sample was mixed with vortex and boiled for 5 min at 100°C on hot plate, then centrifuged at full speed for 1 min, pipetted the supernatant, determined the concentration by spectrophotometry (Nanodrop ND-2000, Wilmington, Delaware, USA) and kept in -20°C until separation. The recipe of lysis buffer was presented below.

Lysis buffer (1 ml)

(i)	Tris-Glycine-Sodium dodecyl sulphate buffer	942 µl
(ii)	Sodium orthovanadate (200mM)	10 µl
(iii)	Protease inhibitor cocktail set1	10 µl
(iv)	Distilled water	38 µl

3.3.5.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used for protein separation. A mini gel electrophoresis system (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) was used with Tris/Glycine buffer which was consisted of 5% stacking gel and 10% separating gel as presented below.

Gel electrophoresis solutions

5% stacking gel solution (4 ml)

(i)	Distilled water	2.2 ml
(ii)	30% acrylamide mix	0.67 ml
(iii)	0.5 M Tris (pH 6.8)	1.0 ml
(iv)	10% Sodium dodecyl sulfate (SDS)	40 µl
(v)	10% Ammonium persulfate (APS)	40 µl
(vi)	TEMED (N,N,N',N'-Tetramethylethylene-diamine)	6 µl

10% separating gel solution (10 ml)

(i)	Distilled water	4 ml
(ii)	30% acrylamide mix	3.3 ml
(iii)	1.5 M Tris (pH 8.8)	2.5 ml
(iv)	10% Sodium dodecyl sulfate (SDS)	100 µl
(v)	10% Ammonium persulfate (APS)	100 µl
(vi)	TEMED (N,N,N',N'-Tetramethylethylene-diamine)	4 µl

Note: APS was prepared freshly before gel preparation. APS and TEMED were added in the last before gel pouring into the glass set.

The separating gel was poured into the glass set (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) (5 x 10 cm per gel). The distilled water was added at the top of separating gel until it set. After the gel has set (approximately 25 min), the water was poured off and followed by the stacking gel pouring. Comb was placed on the top of the stacking gel. The gel was left until setting, rinsed with water, wrapped by humidified paper and kept in 4°C before using.

The extracted protein (10 µg) was mixed with sample buffer (7.25 ml distilled water, 1.25 ml 0.5M Tris pH6.8 , 1ml Glycerol, 0.01g bromophenol blue, 0.2 g SDS, 0.5 ml β-ME) to make the total concentration was 1 µg/µl and loaded into lanes 2 – 8 (one sample per lane). Lane 1 and 10 were loaded with 5 µl of ready-to-used specific molecular weight ladder and lane 9 was loaded with positive control for each protein get from kidney lysate. Electrophoresis was performed at 120 Volt and 450 mA for 45 min

3.3.5.3 Immunoprobing

After gel electrophoresis, the stacking gel was removed and the separating gel was soaked in 1X transfer buffer (100 ml of 10X transfer buffer; Glycine 29.3 g, Tris 51.8 g, SDS 3.75 g made to 1 liter with distilled water; 200 ml of methanol; 700 ml of distilled water). Polyvinylidine Fluoride (PVDF) membrane (PVDF Immobilon-P membrane, EMD Millipore Corporation, MA, USA) was cut into size 5x9 sq. cm. and soaked in absolute methanol until the membrane was transparent, then soaked in distilled water for 5 min and moved to 1X transfer buffer before making blotting layers. The blotting layers were placed in the blotter (Bio-Rad Mini Transblot cells, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) as followed: fiber pad, three filter papers, PVDF membrane, gel, three filter papers and fiber pad. Air bubbles (between

PVDF membrane and gel) were removed by a small bread roller. The blotter layers were placed in the blotter and run at 25 Volt and 50 mA for 60 min.

After blotting, PVDF membrane was stained with 0.1% w/v Ponseau Red in 1% v/v Acetic acid for 5 min to ensure the complete protein transfer, destained 5% v/v acetic acid 5 min for 2 times and then washed with distilled water 5 min for 2 times before blocking. The membrane was cut into two pieces at the above of 75kDal ladder to separate probing of β-actin and interesting proteins, blocked in 10% w/v skimmed milk (10 g of skimmed milk in 100ml of 1X Tris-Buffer Saline-Tween (TBS-T); 10X TBS-T, Tris 60.6 g, NaCl 96.67 g, Tween (0.2%) 2 ml adjusted pH to 7.4 made up to 1liter) and washed by 1X TBS-T at 200 rpm 10 min for 3 times. The membrane under the 75 KDal ladder was used for β-actin probing while the other was used for interesting protein probing. The first membrane was incubated with primary antibody (1:1000 β-actin clone mAb8226, Abcam CL, Cambridge, UK) while the other was incubated with primary antibody as presented below

Table 5 Primary antibody, staining type, brand and standardized dilutions utilized

Primary antibody	Type of staining	Clone	Brand	standardized dilution (IHC)	standardized dilution (WB)
anti-P-gp	cytoplasmic	C494	EMD Milipores	1:50	1:10
anti-MRP1	cytoplasmic	MRPm6	Abcam CL	1:50	1:20
anti-MRP2	cytoplasmic	M_2III-6	Abcam CL	1:50	1:20

After incubation overnight, the membranes were washed with TBS-T on a shaker for 10min X 3 times at 200 rpm. The membranes were incubated with secondary antibody (anti-mouse made in horse; Vector Laboratories, CA, USA) for one hour at dilution (1:10,000 for β-actin, 1:250 for P-gp and MRP1 and 1:500 for MRP2), washed with TBS-T 10 min X 3 times and incubated with

Avidin-biotin method (Vectastain® ABC elite kit, Vector Laboratories, CA, USA) for 30 min. The membranes were washed with TBS-T 10 min X 3 times and submitted to color developing process.

The membranes were incubated with diaminobenzidine (DAB; DAB substrate kit, Vector Laboratories, CA, USA) for 2 min and washed with distilled water 10 min X 2 times. The optical density was calculated using computed analytic software (ImageJ version 1.48, NIH, Maryland, USA) by comparing the level of interested protein with the level of internal control (β-actin).

3.3.6 Statistical analysis

Statistical analysis was carried out using Statistical Analysis Software (SAS version 9.0, Institute, 2002, Cary, NC, USA). Paired *t-test* was used for comparing the differences among weeks of vincristine treatment of mRNA (qPCR) and protein expression (IHC and WB) while Mann-Whitney test was used for comparing between the untreated and the resistance cTVT. The data was presented in mean±SD. Values were considered the significant level at $P<0.05$.

3.4 Results

3.4.1 Experimental I

3.4.1.1 The mRNA expression of MDR1, MRP1 and MRP2 during weeks of treatment

The *MDR1*, *MRP1* and *MRP2* mRNA was detected in all samples of cTVT throughout vincristine treatment. However, there was no significant difference among weeks of treatment. The data was transformed by natural logarithm (Ln of fg/20 ng cDNA) in figure 6.

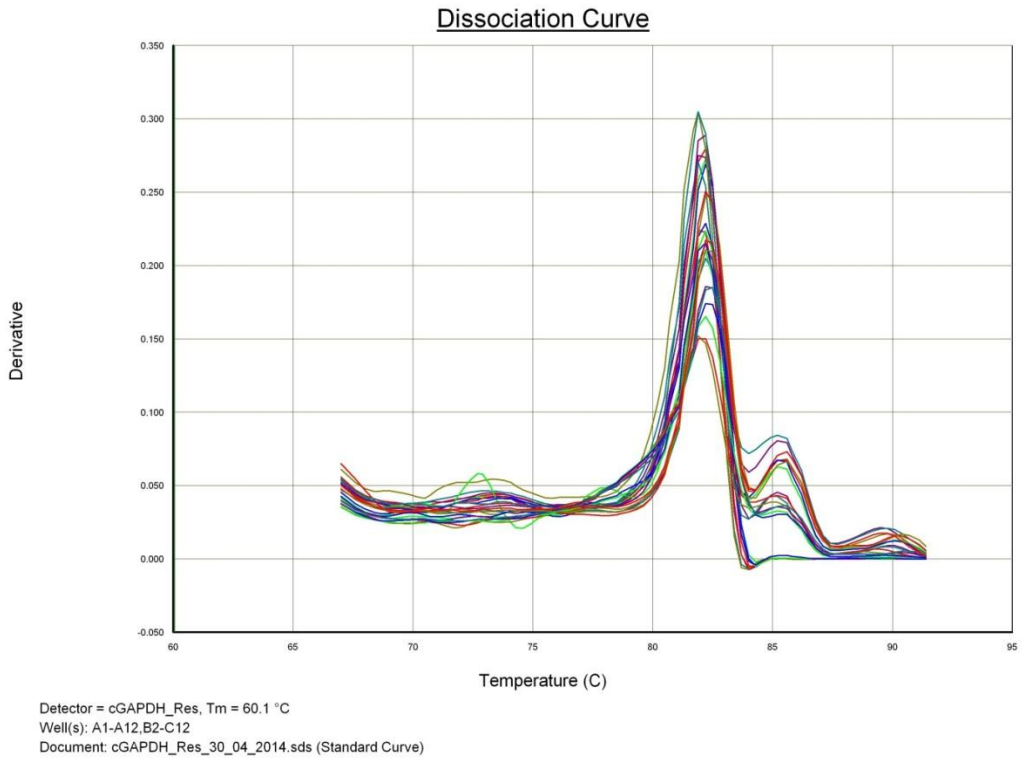

Figure 1 Dissociation curve analysis of *GAPDH* performed at the end of real-time PCR. There was no significant primer-dimers or non-specific products present on the curve.

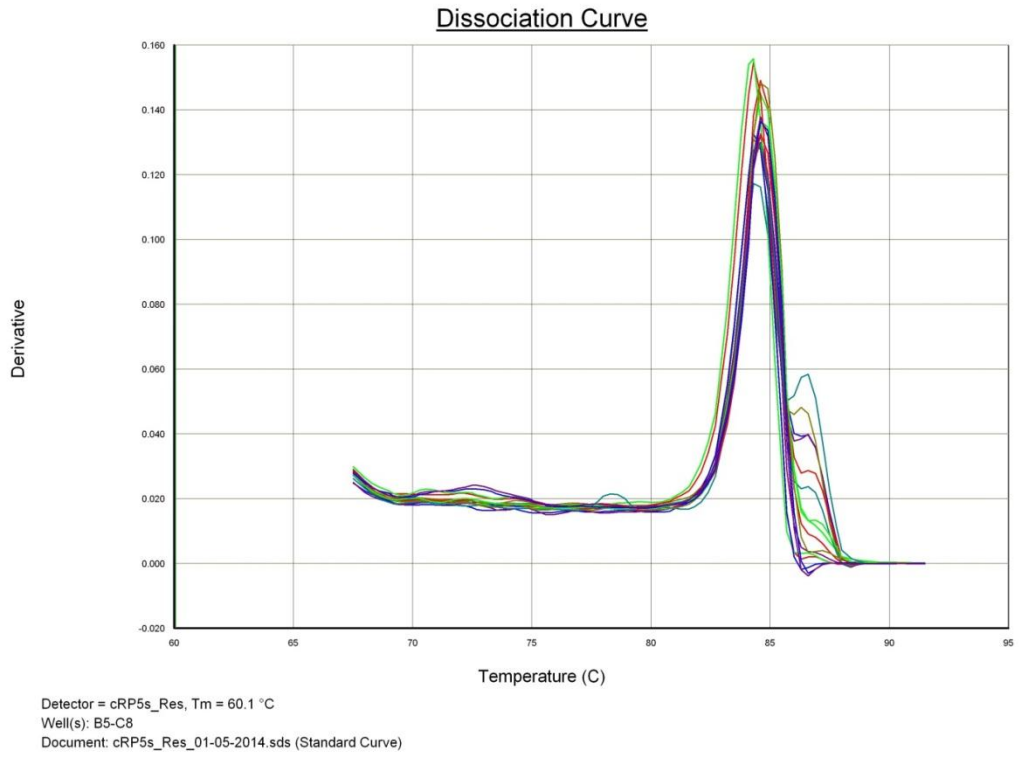

Figure 2 Dissociation curve analysis of *RP5S* performed at the end of real-time PCR. There was no significant primer-dimers or non-specific products present on the curve.

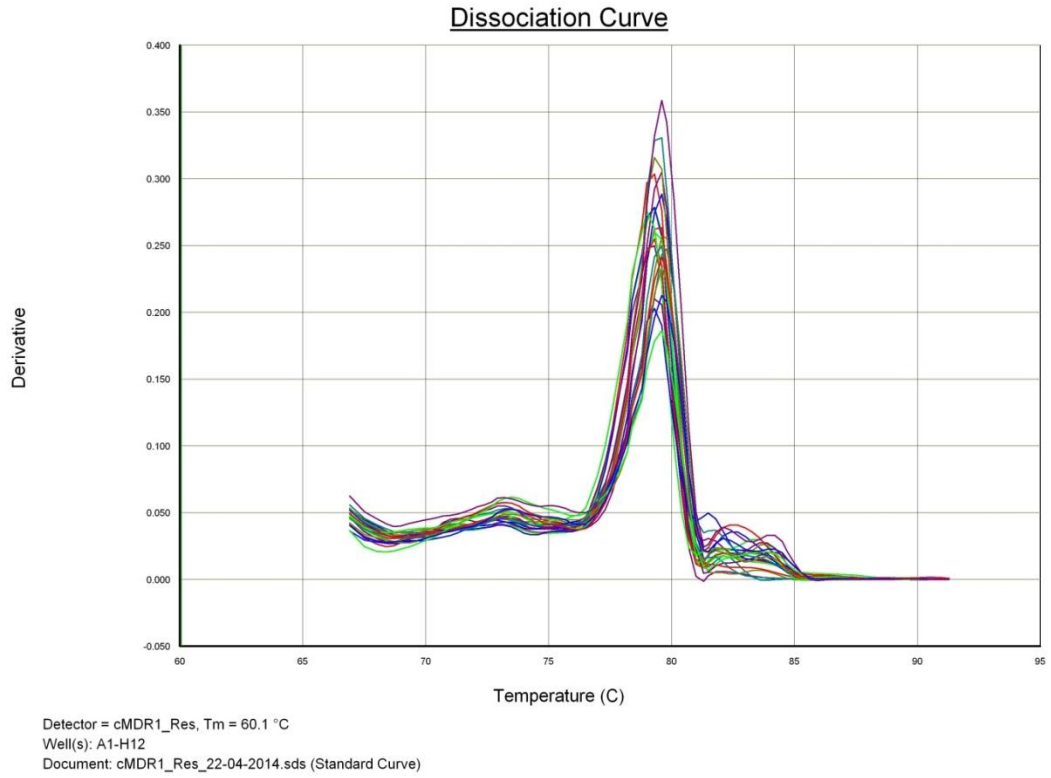

Figure 3 Dissociation curve analysis of *MDR1* performed at the end of real-time PCR. There was no significant primer-dimers or non-specific products present on the curve.

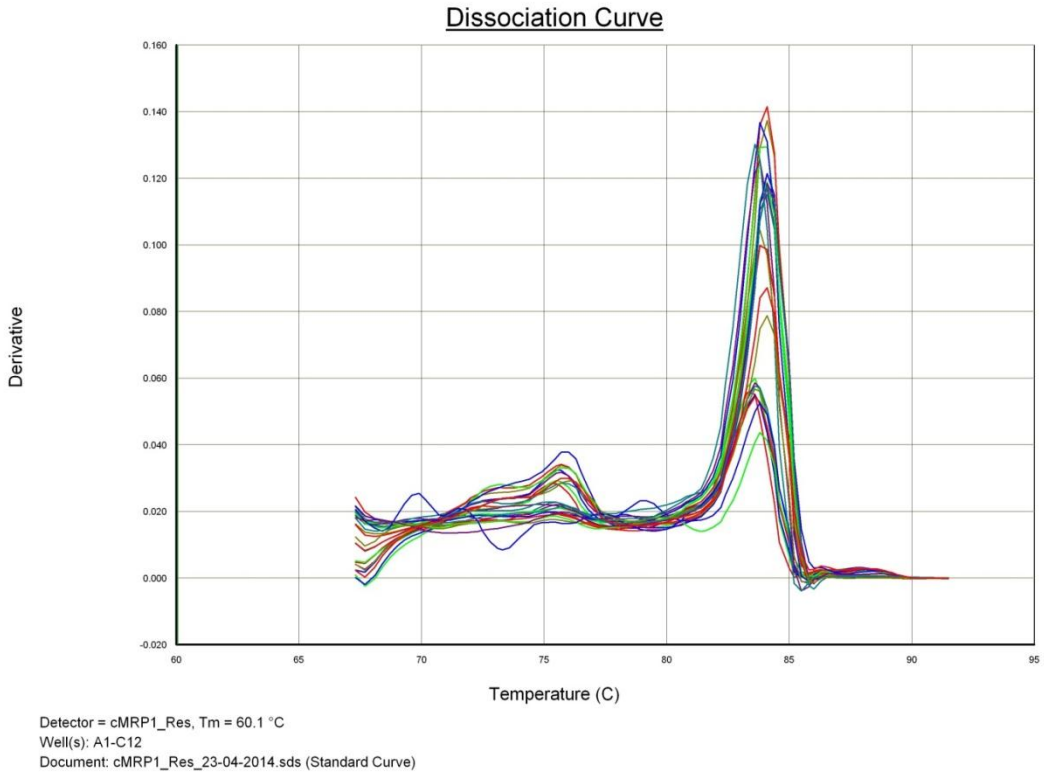

Figure 4 Dissociation curve analysis of *MRP1* performed at the end of real-time PCR. There was no significant primer-dimers or non-specific products present on the curve.

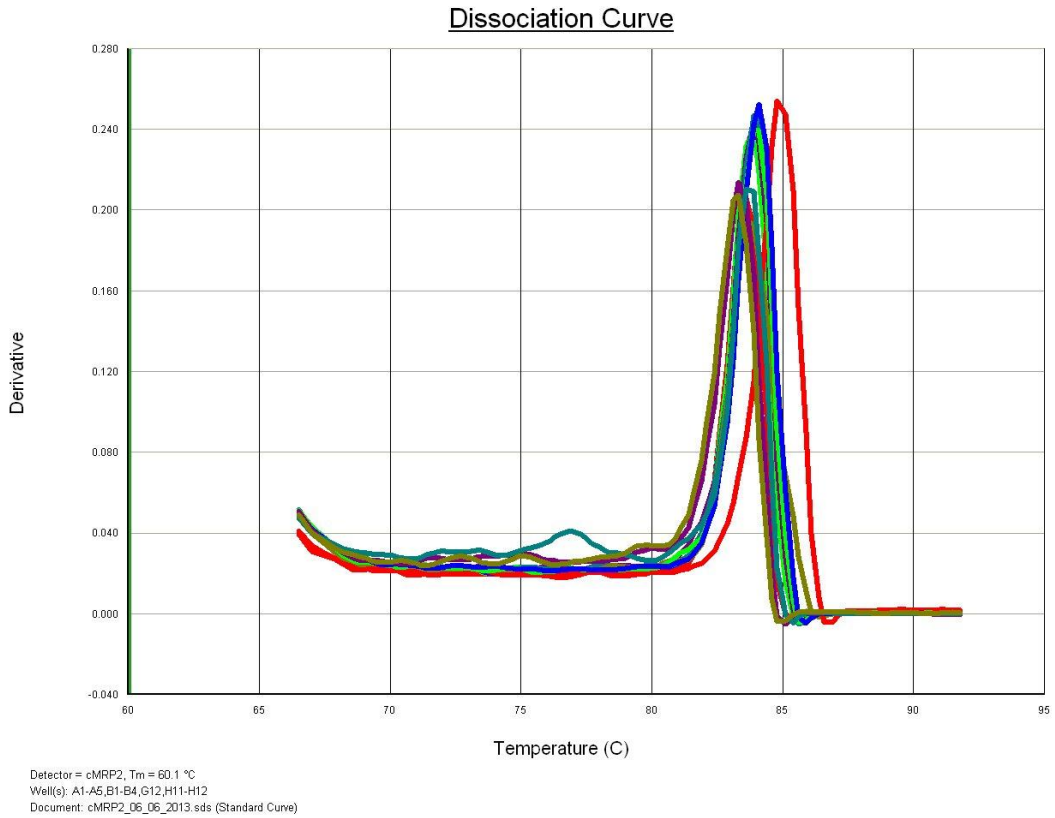

Figure 5 Dissociation curve analysis of MRP2 performed at the end of real-time PCR. There was no significant primer-dimers or non-specific products present on the curve.

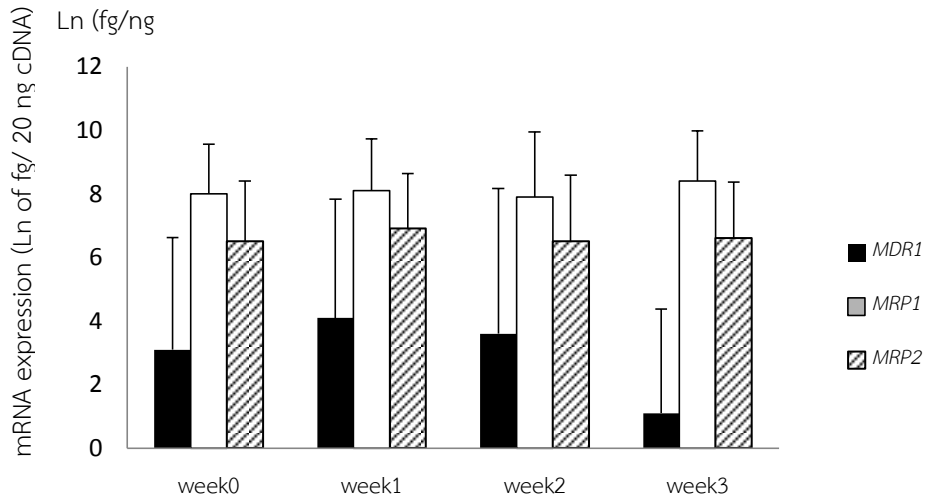

Figure 6 Mean (±SD) mRNA expressions of *MDR1*, *MRP1* and *MRP2* in cTVT during vincristine treatment. Bars with the superscript differed significantly ($P<0.05$)

3.4.1.2 The protein expression of P-gp, MRP1 and MRP2 during weeks of treatment

From cytomorphological study, the cytomorphological pattern was presented in table1. In this study, mixed pattern was predominated in this study (7/12), lymphocytoid pattern (3/12) and plasmacytoid pattern (2/12) while in resistance cTVT revealed cell types as mixed (1/4) and plasmacytoid (3/4). Treatment response in lymphocytoid and mixed pattern showed complete remission on fifth week of treatment while plasmacytoid cTVT revealed regression after 8 weeks of treatment. In 4 resistance cTVT, there was no regression after 8 injections of vincristine.

Table 6 Number of cases (n) and ratio in samples from different cytomorphological appearance of cTVT

groups	cytomorphology	n	ratio
TVT1 (n=12)	lymphocytoid	3	0.25
	plasmacytoid	2	0.17
	mixed	7	0.58
TVT2 (n=4)	lymphocytoid	0	0
	plasmacytoid	3	0.75
	mixed	1	0.25

From immunohistochemistry study, three proteins expressed in all samples of cTVT masses collected during treatment (figure 7-9). P-gp expressed significantly lower in week3 compared with week0 (P=0.026) while there was no significant difference among weeks of treatment in MRP1 and MRP2 protein expression (P>0.05) (figure 10).

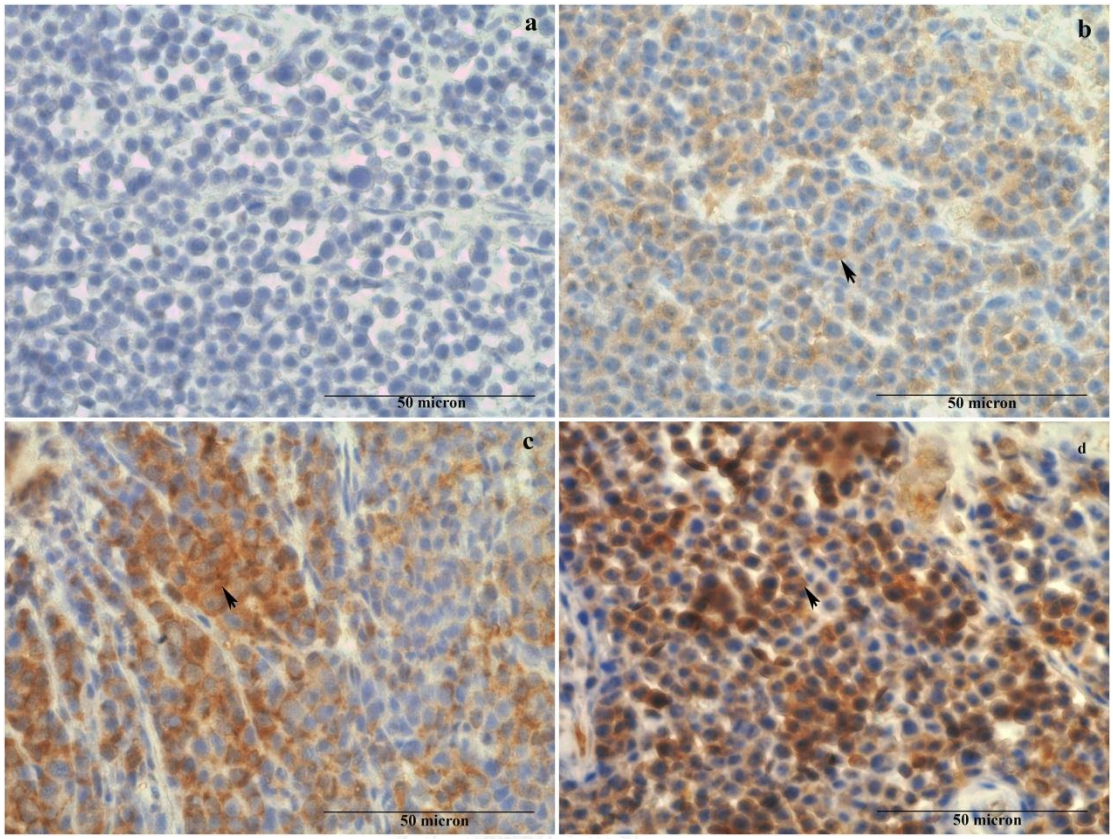

Figure 7 P-gp localization (brown staining) in cTVT mass. Positive staining (arrow) was observed in cytoplasm and cell membrane of cTVT cells. Grading for staining was presented as negative staining (A), moderate staining (B) and intense staining (C, D)

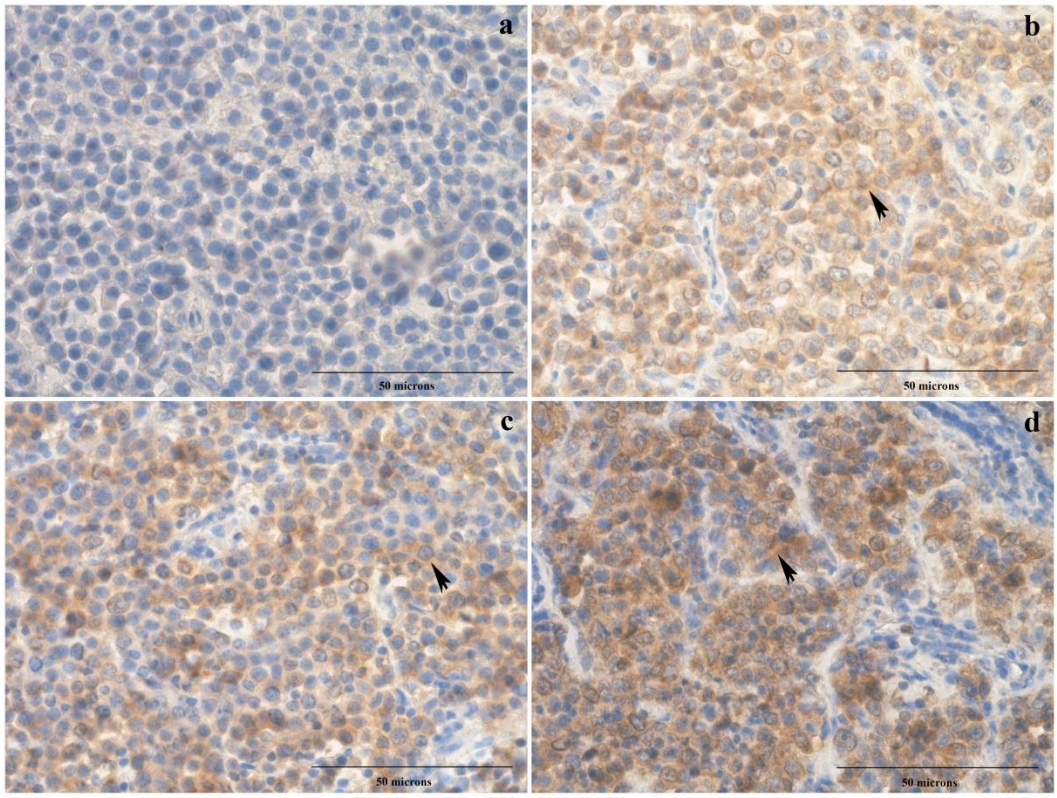

Figure 8 MRP1 localization (brown staining) in cTVT mass. Positive staining (arrow) was observed in cytoplasm and cell membrane of cTVT cells. Grading for staining was presented as negative staining (A), moderate staining (B) and intense positive staining (C, D)

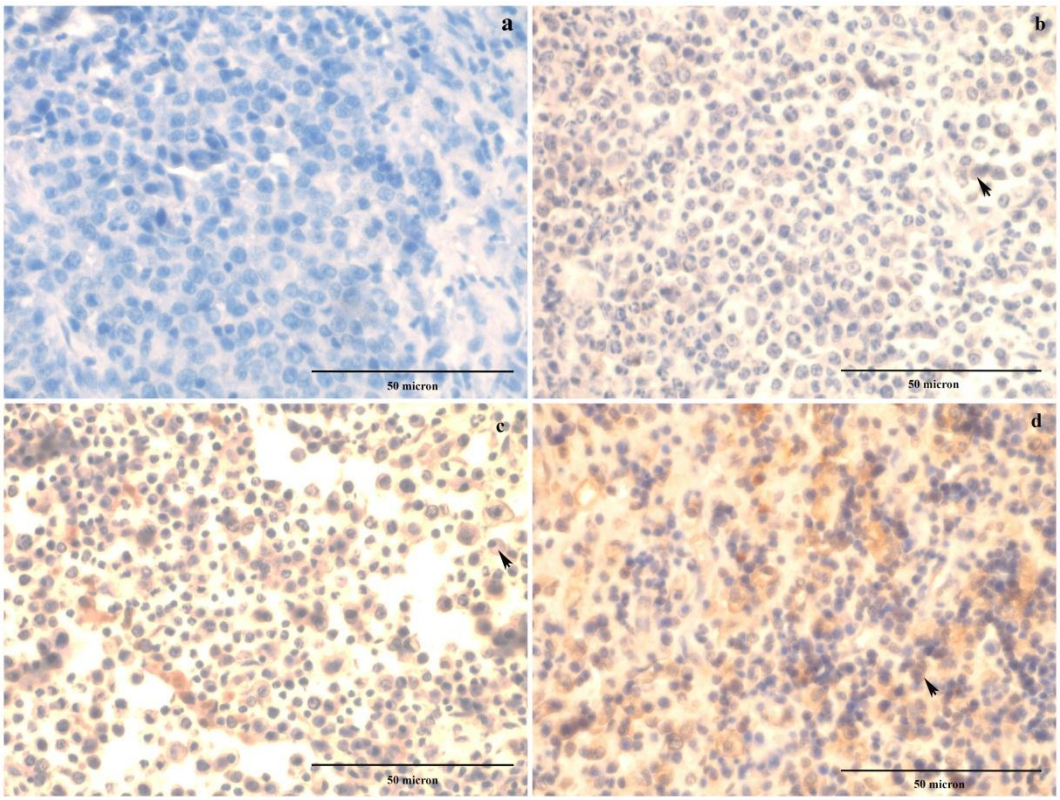

Figure 9 MRP2 localization (brown staining) in cTVT mass. Positive staining (arrow) was observed in cytoplasm and cell membrane of cTVT cells. Grading for staining was presented as negative staining (A), weak positive (B), moderate staining (C) and intense staining (D)

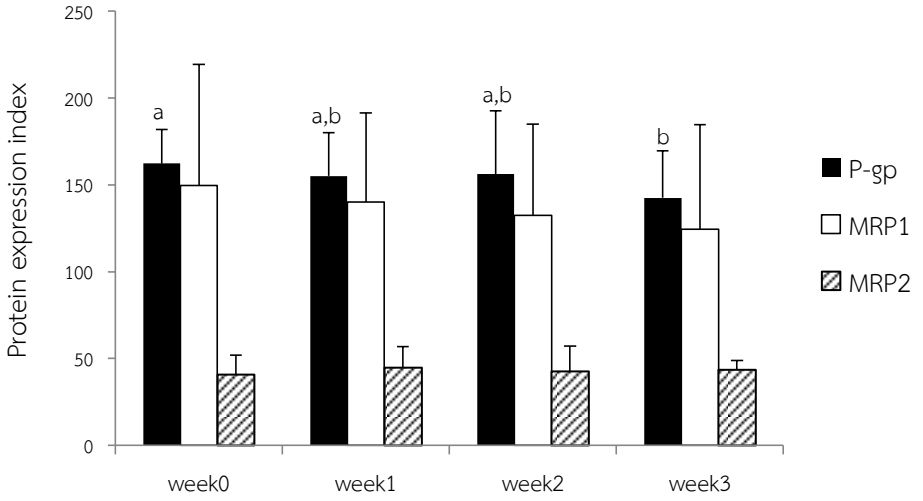

Figure 10 Mean (±SD) expression indexes of P-gp, MRP1 and MRP2 proteins from immunohistochemistry in cTVT during vincristine treatment. Bars with different superscripts show significant differences ($P<0.05$)

3.4.1.3 The protein expression level of P-gp, MRP1 and MRP2 during weeks of treatment

The results from western blot analysis confirmed the protein expression of P-gp, MRP1 and MRP2 from Immunohistochemistry. The presence of protein bands in specific molecular weight was observed in all samples throughout treatment (figure 11) although the significant difference was not observed among weeks of treatment (figure 12).

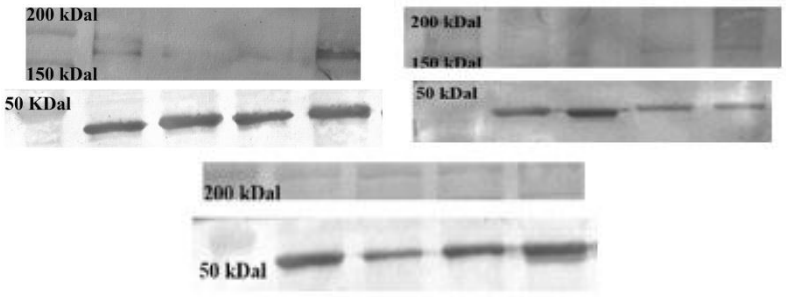

Figure 11 the protein bands at molecular weight approximately 170 kDal (P-gp), 190kDal (MRP1 and MRP2, respectively)

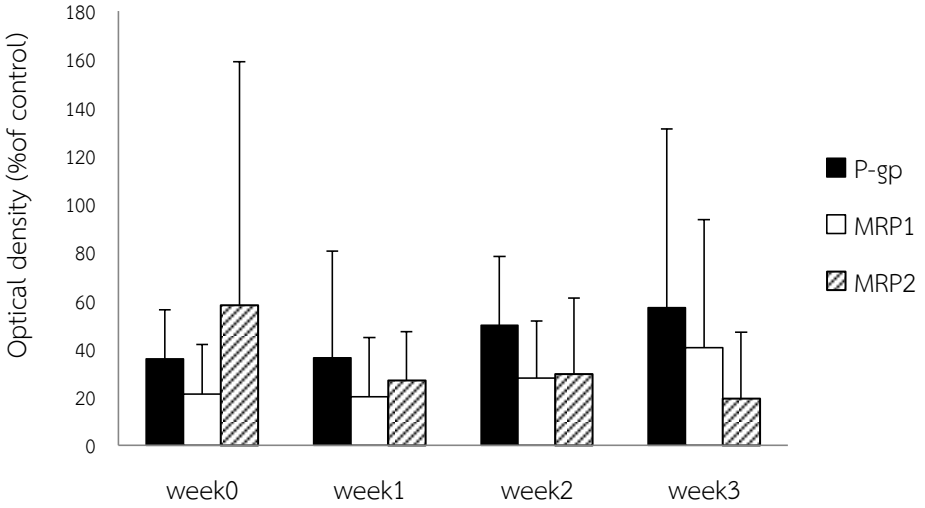

Figure 12 Mean (±SD) optical densities of P-gp, MRP1 and MRP2 from western blot in cTVT during vincristine treatment. Bars with different superscripts show significant differences ($P<0.05$)

3.4.2 Experiment II

3.4.2.1 The mRNA expression of MDR1, MRP1 and MRP2 expression between the untreated and the resistance cTVT

In resistance cTVT cases, the mRNA of MDR1, MRP1 and MRP2 were detected in all samples as well. The mRNA expression of MDR1 was significant lower in the resistance compared with those untreated ($P=0.01$) while the mRNA expression of MRP1 and MRP2 was not significant difference (Figure 13).

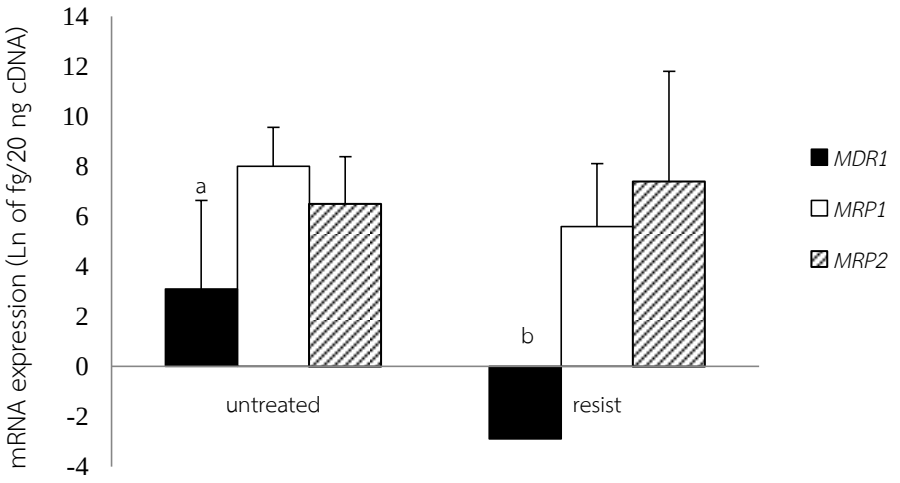

Figure 13 Mean (±SD) mRNA expressions of *MDR1*, *MRP1* and *MRP2* in the untreated and resistance cTVT. Bars with the superscript differed significantly ($P<0.05$)

3.4.2.2 The protein expression of P-gp, MRP1 and MRP2 between the untreated and the resistance cTVT

In resistance cTVT, the P-gp expression was significant lower in the resistance compared with untreated group ($P= 0.02$) while MRP2 expression was significant higher in the resistance ($P=0.03$) but there was no significant difference in MRP1 expression between two groups (Figure 14).

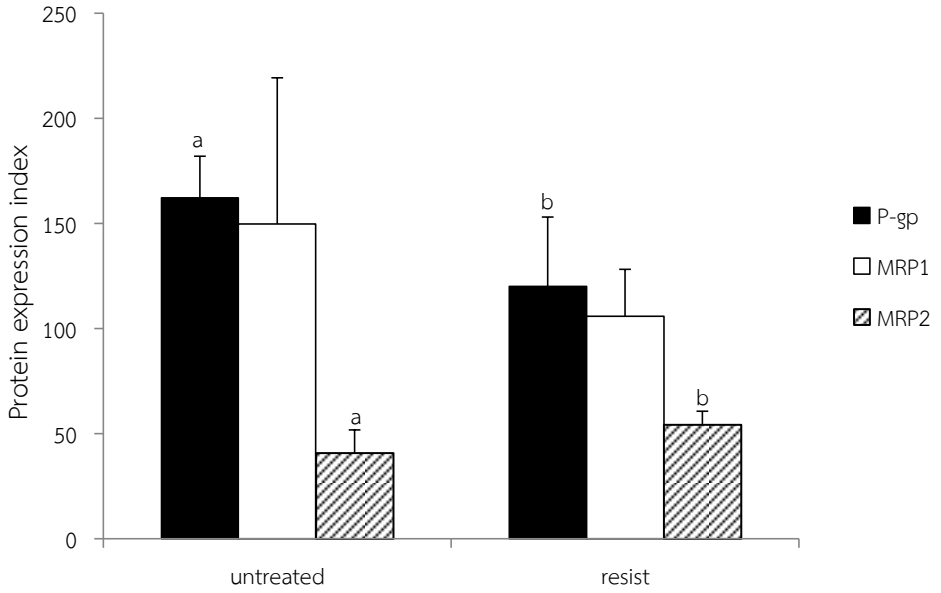

Figure 14 Mean (±SD) expression indexes of P-gp, MRP1 and MRP2 from immunohistochemistry in untreated and resistance cTVT mass. Bars with different superscripts show significant differences ($P<0.05$).

3.4.2.3 The protein expression level of P-gp, MRP1 and MRP2 between the untreated and the resistance cTVT

In resistance cTVT, the protein bands were presented in specific molecular weight. The optical density of protein expression revealed the P-gp expression was lower in the resistance ($P=0.02$) while MRP1 and 2 were not significant difference between the resistance and untreated groups (figure 15).

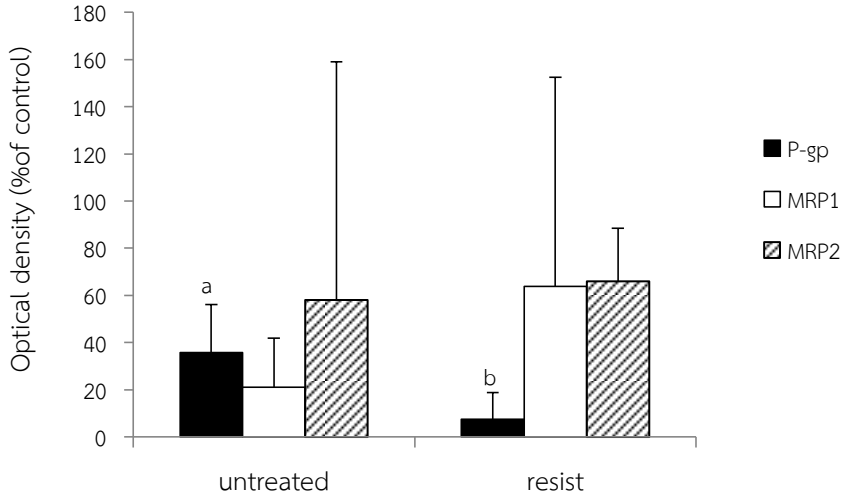

Figure 15 Mean (±SD) optical densities of P-gp, MRP1 and MRP2 from western blot in untreated and resistance cTVT mass. Bars with different superscripts show significant differences ($P<0.05$)

3.5 Discussion

The commonly used chemotherapy for cTVT treatment is vincristine yielding 90% complete remission (Calvert et al., 1982; Rogers et al., 1998; Gerardi et al., 2014). However, the resistance has been reported in many studies (Rogers et al., 1998; Said et al., 2009; Gaspar et al., 2010; Kunakornsawat et al., 2010; Stockmann et al., 2011). The resistance mechanism is still unclear but researchers believe that ABC-transporters may be involved especially P-gp (Gaspar et al., 2010; Stockmann et al., 2011). Previously, the studies focused on the expression of P-gp in cTVT which could excrete and decrease intracellular cytotoxic drug concentration led to the survival of tumor cells and the presence of refractory effect from vincristine treatment (Gaspar et al., 2010; Stockmann et al., 2011). Recently, cytomorphological study arose in the study of Santos de Amaral et al. (2007) that revealed the pattern of cTVT cellular characteristics as lymphocytoid pattern; small cell size with round concentric nucleus, plasmacytoid pattern; large cell size and abundant cytoplasm with

eccentric nucleus and mixed pattern; mixed population of lymphocytoid and plasmacytoid cell types. In this study, TVT1 group revealed mixed pattern and TVT2 showed plasmacytoid pattern were predominated (in Table 6) which was similar to the previous study (Gaspar et al., 2010). In TVT1, cytomorphological pattern showed good response to vincristine treatment while the resistance was found in TVT2. This finding might be explained that plasmacytoid pattern may relate to vincristine resistance as mentioned in the previous study (Gaspar et al., 2010). In this study, the ABC-transporter gene expression showed the difference in both groups. In TVT1, there was no significant difference of *MDR1*, *MRP1* and *MRP2* mRNA expression during weeks of treatment differed from many studies (Nakaichi et al., 2007; Honscha et al., 2009; Zandvliet et al., 2014) which expressed increasingly during treatment. In TVT2, the *MDR1* mRNA expression expressed lower in resistance group compared with untreated group while *MRP1* and *MRP2* mRNA expression were not significant difference (Figure 7). From the study of Hsiao et al. (2004) suggested that cTVT remission differed from the other tumor. In regression phase of cTVT, tumor-infiltrating lymphocytes (TILs) play the special roles in cTVT mass by releasing interleukin-6 (IL-6) that increase MHC class I expression, counteract transforming growth factor-β1 (TGF-β1) and restore the cytotoxic activity of CD8+ T-lymphocytes (Hsiao et al., 2004). This scheme was found in experimental inoculating and transmitted cTVTs (Gonzalez et al., 2000). The study of Sukhai et al. (2001) revealed the decrease in P-gp expression in rat hepatocytes from IL-6 treatment and this finding was found the effect of IL-6 on P-gp expression in human hepatocytes (Fernandez et al., 2004). This might be explained the lowering in *MDR1* mRNA expression in TVT2. In protein expression from both IHC and WB, significant reduction in P-gp expression between untreated and week3 after treatment (TVT1) and the reduction of P-gp expression between

untreated and resistance (TVT2) might be resulted from IL-6 releasing. The vincristine treatment may trigger some mechanisms in cTVT cells led to cellular morphology presented in TVT2 was mixed and plasmacytoid than lymphocytoid. Pattern of P-gp expression in tumor was classified as 4 categories. First, tumors always express P-gp; second, tumors express P-gp sometimes; third, tumors rarely express P-gp and fourth, tumors express P-gp after chemotherapy induction (Gaspar et al., 2010). Gaspar et al. (2010) suggested that P-gp expression pattern might be the first or second. This finding might be explained that the reduction of lymphocytoid cell type responded to IL-6 releasing during vincristine treatment caused lowering P-gp expression (Figure 11 and 12). The expression of MRP2 from IHC showed significant higher in resistance cTVT might be the mechanism that cTVT cells used for survival through vincristine treatment that was similar to the mechanism in human pancreatic cancer showed the increase in MRP2 expression after treatment (Noma et al., 2008). From the study of Ramji and Foka (2002) revealed that IL-6 regulated the P-gp and MRP2 expression in pre-transcription process via Nuclear factor-**K**B (NF-**K**B) and CCAAT/Enhancer Binding proteins (C/EBPs). These transcription factors were found in the pre-transcriptional area of *MDR1* and *MRP2* genes. The study of Dos Santos et al. (2010) suggested that vincristine treatment triggered NF-**K**B signaling pathway that inhibited the transcription of *MDR1* gene leaded to inhibited P-gp translation causing decrease in P-gp production while Ramji and Foka (2002) revealed that IL-6 induced C/EBPs which triggered MRP2 transcription and led to the increase in MRP2 production. From this finding, MRP2 might relate in resistance process than P-gp and MRP1. This evidence was supported by the study of Gerardi et al. (2014) which showed the expression of glutathione-S-transferase in resistance cTVT that

MRPs used glutathione for cytotoxic drug co-transportation (Gottesman et al., 2002).

In conclusion, ABC-transporter gene and protein expression might be the support information for resistance prediction in chemotherapy which expressed differently depending on the type of tumor. The different types of tumor would express different protein expression pattern. The protein expression might be involved in cytomorphological pattern which helps in resistance prediction and MRP2 might be involving in cTVT resistance mechanism.

Chapter IV

Treatment of canine transmissible venereal tumor using vincristine sulfate combined with L-asparaginase in clinical vincristine-resistance cases: a case report

4.1 Abstract

Three female mongrel dogs were diagnosed as transmissible venereal tumor (TVT) and had clinically developed resistance to vincristine treatment. One dog was treated with 10,000 IU/m^2 body surface area of L-asparaginase combined with 0.025 mg/kg body weight of vincristine sulfate every two weeks for 4 treatments while the others were administered this combination once weekly for four weeks and continued with only vincristine sulfate once weekly for four treatments. Both treatments resulted in a complete remission. Side effects such as gastrointestinal upset, diarrhea, depression, and decrease in appetite were observed in the dogs administered with the later protocol. Hematologic disturbance was observed in one out of two dogs showing leukopenia three weeks after the treatments. Although the complete regression of tumor was observed in both treatment courses, two-week treatment interval is recommended to avoid undesirable effects.

4.2 Introduction

Naturally occurring canine transmissible venereal tumor (TVT) is an important contagious neoplasm commonly attacks the reproductive tract. This tumor widely spreads in free-roaming dogs (Rogers et al., 1998; Ganguly et al., 2013). It is classified into two groups; genital TVT and extragenital TVT, according to the locations of the tumor mass present (Das and Das, 2000). Genital TVT is transmitted via natural mating while extragenital TVT is occurred by social contact, like sniffing or licking (Otomo et al., 1981). Prevalence varied upon the areas, for example; 11% in Kenya, 32% in Sri Lanka, 10% in Marryland (USA) and 23.5 to 28.6% in India (Das and Das, 2000). The clinical presentations for TVT are visible cauliflower-like mass in genital area or on skin surface with the presence of bloody discharge, ocular or nasal deformation from tumor invasion (Rogers et al., 1998; Mello Martins et al., 2005). Cytological method is commonly used to diagnose the tumor because it is easy, less painful and less time consuming than biopsy (Santos de Amaral et al., 2007). Treatments used to cure TVT are surgery, radiation or chemotherapy. Surgical tumor removal does not only provide unsatisfactory response but also causes tumor recurrent. Although, radiotherapy yields complete regression, it requires trained workers, special equipments and expenses (Boscos and Ververidis, 2004). However, chemotherapy yields similar good response and tumor regression to radiotherapy. Vincristine sulfate has been widely accepted as an efficient single chemotherapeutic agent for treatment of TVT (Mello Martins et al., 2005). Vincristine sulfate acts by binding to tubulin dimer which is necessary for mitosis of spindle fibers, contributing to cellular division arrested in metaphase stage (Coppoc, 2009). The typical course of vincristine treatment is four to eight weekly intravenous administrations at 0.5 to 0.7 mg/m^2 body surface area (BSA) (Boscos and Ververidis, 2004) or 0.025 mg/kg body weight (BW) (Das and

Das, 2000). TVT remission can be classified into complete response (CR), characterized by total regression of the tumor mass; partial response (PR), showed regression of more than 50% of the tumor mass; and no response (NR), defined as less than 50% regression (Rogers et al., 1998). The non-responsive vincristine cases have been occasionally reported (Rogers et al., 1998; Das and Das, 2000) which are suggested to receive alternative treatments, such as radiotherapy (Rogers et al., 1998; Boscos and Ververidis, 2004), surgery (Kunakornsawat et al., 2010), and other chemotherapeutics such as doxorubicin, vinblastine, methotrexate, predinisolone or cyclophosphamide as a single or in combination between 2 to 3 drugs. However, side effects usually occur when the combined chemotherapeutics are used and recurrence is seen in cases treated by surgical removal (Das and Das, 2000; Boscos and Ververidis, 2004; Kunakornsawat et al., 2010). L-asparaginase is one of the chemotherapeutic agents used for pediatric acute lymphoblastic leukaemia (ALL) and lymphoma in human (Narta et al., 2007). It has been applied also to treat canine leukaemia, lymphoma (Barton, 2001) and cutaneous lymphoma (Theewasutrakul et al., 2007). Asparagine is a non-essential amino acid, synthesized in normal cells by enzyme asparagine synthase. L-asparaginase acts by reducing asparagine pool which is required for cellular proliferation and differentiation of tumor cells (Muller and Boos, 1998; Barton, 2001). However, the tumor was not regressed totally when the L-asparaginase was administered as single chemotherapeutic in canine cutaneous lymphoma whereas the total regression was observed when vincristine sulfate was accompanied (Theewasutrakul et al., 2007). Thus, these two chemotherapeutics might worth be tested also in non-responsive TVT cases in this study.

4.3 Case history

History of three dogs diagnosed as TVT was summarized in Table 7. The dogs had been treated with vincristine sulfate at 0.025 mg/kg body weight once weekly. Case I was an intact female mongrel dog presented with vaginal tumor. She had been treated with vincristine sulfate intravenously once weekly. After six months, the tumor was recurred at the vulva area. A new treatment course was started but the tumor regressed partially. Then she was referred.

Case II was an intact female miniature pincher-cross breed dog. The TVT masses were completely regressed after treatments with vincristine sulfate. Two years later, she presented with irregular vulval mass with ulcerative and deformed vulva. Treatment was started with vincristine sulfate once weekly. The tumor regressed gradually but the visit was skipped thereafter. Thereafter, she was re-introduced but the treatment with vincristine sulfate alone was not cured. Thus, she was enrolled in this program.

Case III was a spayed female miniature pincher-cross breed dog. She had been treated with vincristine sulfate intravenously once a week until the tumor regressed totally. After two months, a bloody vaginal discharge and vaginal mass were presented (Figure 16). A new treatment course was started but leukopenia was noticed. Therefore, she was referred.

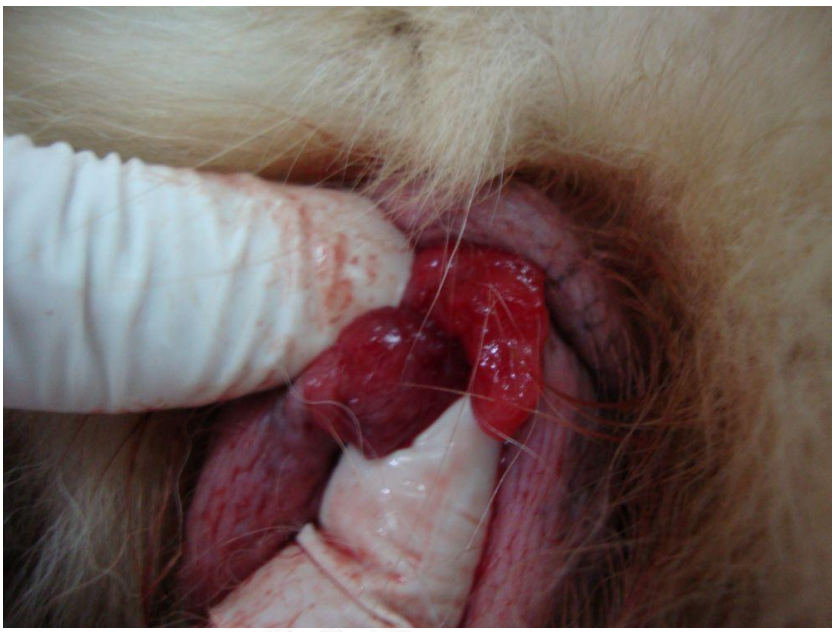

Figure 16 Venereal tumor mass covered with bloody discharge in the dorsal wall of the vagina of bitch case III (mixed breed dog) on first day before treatment.

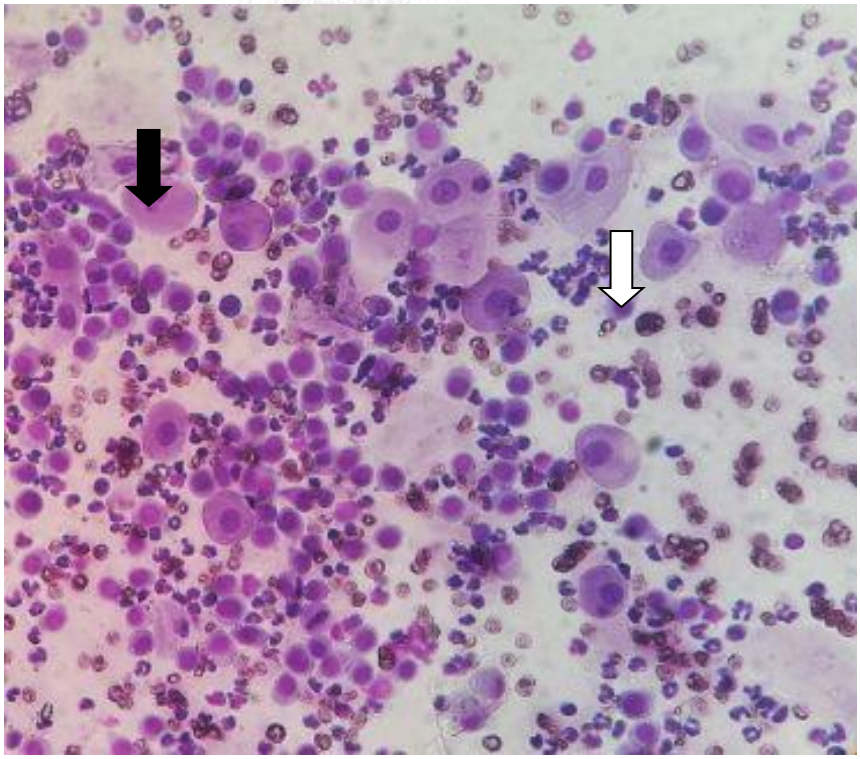

Figure 17 Plasmacytoid cell-type of the canine transmissible venereal tumor smeared from the vagina of a bitch (black arrow). Many of white blood cells were presented (white arrow).

Table 7 History of individual dogs prior to combination treatment submitted

Case number	Gender	Genital TVT	Extra-genital TVT	Interval before previous course	Numbers of treatment	Results
1	Female	vagina	-	-	Course 1: treated by vincristine for 4 injections	Complete regression
		vulva	-	6 months	Course 2: treated by vincristine for 10 injections	Partial regression with bloody vaginal discharge was observed.
2	Female	vagina	orbit	-	Course 1: treated by vincristine for 2 injections	Complete regression of vaginal mass and ocular mass
		vagina	right upper eye lid	3 months	Course 2: treated by vincristine for 5 injections	Complete regression of vaginal mass after four injections and total regression of all tumors after five injections. Vaginal mass was observed two years after the last session of treatment.
3	Female	vagina	-	-	Course 1: treated by vincristine for 5 injections	Partial regression of vaginal mass.
		dorsal vagina	-	3 months	Course 2: treated by vincristine for 8 injections	Non-response was observed after 8 injections and side effects were presented as leucopenia. Then she was referred to CU-Vet Small Animal Hospital.

Table 8 Results after combination treatment

Case number	Previous treatment outcome	Interval from last treatment	Treatment course	Treatment outcome	Side effect
1	Partial response	3 months	vincristine 0.025 mg/kg BW with L-asparaginase 10,000 IU/m^2 BSA every 2 weeks for four treatments	Complete remission	Not found
2	Non response	2 years	vincristine 0.05 mg/kg BW with L-asparaginase 10,000 IU/m^2 BSA once weekly for four treatment and followed by vincristine 0.05mg/kg BW once weekly for four treatments	Complete remission	Soft feces was found three days after treatment
3	Non response	4 months	vincristine 0.025 mg/kg BW with L-asparaginase 10,000 IU/m^2 BSA once weekly for four treatment and followed by vincristine 0.025mg/kg BW once weekly for four treatments	Remnant remission and disappear after six months	Soft feces and inappetite was observed three days after treatment. Leucopenia (2400 cells/µl) was observed three weeks after treatment.

4.3.1 Diagnosis and treatment

All three cases were referred to the Small Animal Teaching Hospital at Faculty of Veterinary Science, Chulalongkorn University. They were diagnosed as TVT by exfoliated cell cytology. The samples were smeared and stained with a commercial modified Giemsa staining (Diff-Quick®, S.E. Supply, Bangkok, Thailand). The cytology showed round-to-oval shaped cells with increased ration between nucleus and cytoplasm, dense nucleolus and intracytoplasmic vacuoles suggesting TVT (Figure 17). All cases were treated with vincristine sulfate at private clinics once weekly more than 5 times and still found tumor mass and cells before enrolled combined treatment. Hematological and blood chemistry profile including blood urea nitrogen, creatinine, alanine aminotransferase and alkaline phosphatase were analyzed and defined as in normal range before the treatment was started. Blood samples were collected every two weeks for hematological and blood chemistry profile during treatment program.

In case I, the treatment was started with vincristine sulfate (Vincristin®, Gedeon Richter, Hungary) at dosage of 0.025 mg/kg body weight (BW) intravenously and L-asparaginase (Leunase®, Kyowa Hakko Kogue, Japan) at dosage of 10,000 IU/m^2 body surface area (BSA) every two weeks for four treatments. Two weeks after the first treatment, the tumor size was regressed more than 50% and blood profile did not show abnormality. Thereafter, 90% of the tumor mass regressed after the second treatment. After the third treatment, cytological finding revealed no round cell characterized TVT cells. The dog was followed up by physical examination and cytological method after two and six months after treatment. The tumor recurrence was not observed.

Case II was treated with vincristine sulfate (Vincistin®, Gedeon Richter, Hungary) at dosage of 0.05 mg/kg BW intravenous injection and l-asparaginase (Leunase®, Kyowa Hakko Kogue, Japan) at dosage of 10,000 IU/m^2 BSA once weekly for four treatments and continued with vincristine sulfate at the same

dosage once weekly for four treatments. Prior to L-asparaginase was given, chlorpheniramine maleate was injected intramuscularly at 4 mg/dog to reduce allergic signs. Two weeks after the first treatment, the dog showed clinical signs of gastrointestinal aberrant as mild diarrhea and reduced appetite but the signs were recovered before the next treatment. Tumor size was regressed to more than 50% after three weeks form the first injection and regressed completely on the eighth week of treatment. There was no recurrence observed during six months monitoring since the last injection.

Case III was started with vincristine sulfate (Vincistin®, Gedeon Richter, Hungary) at dosage of 0.025 mg/kg BW intravenously and L-asparaginase (Leunase®, Kyowa Hakko Kogue, Japan) at dosage of 10,000 IU/m^2 BSA once weekly for four injections and followed by vincristine sulfate at 0.025 mg/kg BW once weekly for four injections. Chlorpheniramine maleate was given at 4 mg/dog intramuscularly fifteen minutes before l-asparaginase administration. The tumor size was regressed to 50% in one week after the first injection. Side effects such as soft feces, reduced appetite and leucopenia, were observed at week 3 after the first injection. After the treatment course was ended, the tumor size was persisted at 0.3 cm in diameter and the tumor disappeared two months later. There was no recurrence observed at six months after last treatment. All treatment courses and outcomes are summarized and presented in table 8.

4.4 Results and Discussion

L-asparaginase is a common chemotherapeutic agent widely used in human especially in children acute lymphoblastic leukaemia (ALL). L-asparaginase hydrolyses asparagine to aspartic acid and reduces serum asparagine concentration. This mechanism causes depletion of asparagine which is necessary for protein biosynthesis (Müller and Boos, 1998). The restriction of asparaginase activity in tumor cells causes depletion of asparagines which is necessary for protein synthesis, leads to tumor regression (Capizzi et al., 1970; Muller and Boos, 1998). Side effects are the important

concern for selecting type of chemotherapeutic usage. The side effects observed in every two-week treatment course were less than that treated every one week. Previous study demonstrated the side effects of vincristine at the dosage of 0.5 mg/m^2BSA, such as decreasing in appetite, diarrhea and diffuse alopecia (Said et al., 2009). However, the same side effects were not observed in the study of Kunakornsawat et al. (2009) in which the vincristine sulfate was given with a higher dosage at 0.7 mg/m^2 BSA. In this study, the side effects occurred in the two cases after the second treatment were similar to the study of Tuntivanich (1983) that in which the vincristine sulfate was administered in combination with methotrexate for the TVT treatment.

In general, the vincristine sulfate yields a good response in TVT cases (Rogers, 1997; Rogers et al., 1998; Mello Martins et al., 2005; Said et al., 2009). With single chemotherapeutic use the TVT regression occurs after four to six injections (Boscos and Ververidis, 2004; Said et al., 2009). A resistance may be implied if the regression is not achieved after the sixth injection (Said et al., 2009). In this study, all of the three cases had received up to six injections of the vincristine sulfate with failure of tumor regression; therefore, they were postulated as resistance cases. Interruption of the treatment and duration of the development of the tumor mass likely were the causes of the resistance (Boscos and Ververidis, 2004). In this study, the dogs had the history of vincristine discontinuation during treatment which might contribute to a development of TVT resistance. Drug interruption induced resistance was confirmed by the previous study demonstrating the lower administration of anti-neoplastic drug in TVT tumor cell culture, resulting in the survive cells and expand cell line (Rumjanek et al., 2001; Hirose, 2002; Sulova et al., 2009). Moreover, resistance to chemotherapy may be associated with failure of drug accumulation in neoplastic cells by increasing in drug efflux or decreasing in drug influx controlled by the cell transporters (Gottesman et al., 2002). P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) are transmembrane transporters causing drug elimination from normal and

neoplastic cells. These two proteins play the major roles on neoplastic drug resistance in both human and animal. Recently, many researchers believed that the TVT resistance may be associated with these two proteins (Gaspar et al., 2010) but the mechanism is still unclear.

In conclusion, the combination of L-asparaginase and vincristine is suggested as an alternative treatment for vincristine resistance TVT cases. The appropriate protocol is recommended as being given every two weeks in order to avoid side effects.

CHAPTER V

General Discussion and conclusion

Chemotherapy is an effective option for cancer treatment yielding a good response but drug resistance has been discovered for decades, which is the major obstacle in cancer treatment both in human and veterinary oncology. In small animal oncology, canine transmissible venereal tumor (cTVT) is a common contagious immune-related tumor in the reproductive tract in which vincristine yields 90% of treatment response. However, the resistance has been documented. Although the resistance mechanism is still unclear but the researchers believe that P-gp might involve (Gaspar et al., 2010; Gerardi et al., 2014). This present study revealed exogenous (host factors, tumor factors and environmental factors) as well as endogenous (gene and protein expression) factors affecting outcome during treatment.

It appeared that the tumor size, tumor location and maxTemp60 during vincristine treatment influenced the tumor remission. The results (Chapter II) showed that host general background; composed of age, breed and gender did not affect the treatment outcome. The older cases required more times of injection than the younger ones (Scarpelli et al., 2010) which differed in this study. Breed and gender did not effect cTVT treatment which is similar to the previous studies (Scarpelli et al., 2010; Ganguly et al., 2013). Our findings demonstrated that tumor characteristics (size and location) influenced the treatment outcome. Larger tumors required more vincristine injections than smaller ones. It might be that the large tumors produced the larger amount of immunosuppressive factors such as TGF-β1 that could suppress MHC class I expression thereby retarded tumor clearance (Hsiao et al., 2008; Scarpelli et al., 2010). The other factor that impacted the treatment outcome was the tumor location with the genital cTVT showed a better response to vincristine than extra-genital cTVT or mixed type. This finding might be explained by the previous study demonstrating that predominated cell type in cTVT might be

involved (Fonseca et al., 2012). The predominated cell type of the genital cTVT was lymphocytoid which yielded a better response to vincristine treatment than the extra-genital cTVT in which plasmacytoid was predominated (Fonseca et al., 2012). Moreover, there was a tendency that partial remission occurred in the cases treated during higher mean temperature condition. It was likely that the warmer climate condition affected the host immunity, thereby retarded the tumor clearance. This finding was similar to the study by Scarpelli et al. (2010) showing that more injections of vincristine were required in the hot and wet condition than the cool and dry condition. The host immune function was compromised by the high ambient temperature resulting in reduced lymphocyte function in the cows (Lacetera et al., 2005). Moreover, the chronic exposure of the high ambient temperature induced corticosteroid release contributing to the decrease in lymphocyte activity (Hekman et al., 2014). Therefore, the decrease in lymphocyte activity likely affected the tumor cell elimination in this study.

The cytomorphological study of cTVT and treatment outcome led to the next part of this thesis where the partial remission in the vincristine resistance cases was focused. In cTVT, there were the studies of drug resistance mechanism demonstrating that the expression of ATP-binding cassette (ABC) transported cytotoxic drugs into extracellular area and kept the tumor cells survived through the treatments (Gaspar et al., 2010; Gerardi et al., 2014). However, the resistance mechanism is still unclear. In the previous study, the resistance in cTVT usually occurred in the tumor containing plasmacytoid cell type which had a relation with P-gp expression (Gaspar et al., 2010). In Chapter III, the expression of *MDR1* gene that produced P-gp expression was found in all cTVT samples throughout the treatments but the *MDR1* mRNA expression was not significantly different among weeks of the treatments. It was suggested that *MDR1* mRNA expressed intrinsically before treatment but was not induced to increase during the four weeks of treatment. The intrinsic expression of P-gp might result from the mutation of *TP53* gene which was related to the

apoptotic mechanism (Stockmann et al., 2011). This evidence was observed in many types of the tumors including cTVT (Stockmann et al., 2011). However, the decrease in P-gp expression in cTVT during treatments in this study might be resulted from the increase of interlekin-6 (IL-6) that occurred during cTVT regression. Canine TVT is a unique tumor differs from others in that host immunity plays an important role in tumor regression (Hsiao et al., 2008). IL-6 was secreted from tumor-infiltrating lymphocytes to inhibit TGF-β1 and increase MHC class I expression leading to tumor regression (Hsiao et al., 2004; Hsiao et al., 2008). IL-6 has been shown to cause a decrease in both P-gp mRNA and protein expression in rat hepatocytes (Fernandez et al., 2004). During the treatments, the MRP1 mRNA and protein expression was not significantly different among weeks of the treatment. The regulation of P-gp and MRP2 expression by IL-6 in pre-transcription process has been described via Nuclear factor-**K**B (NF-**K**B) and CCAAT/enhancer binding proteins (C/EBPs) (Ramji and Foka, 2002). These transcription factors were necessary for the pre-transcriptional process of MDR1 and MRP2 genes. This study suggested that vincristine treatment triggered NF-**K**B signaling pathway that inhibited the transcription of MDR1 gene leading to P-gp translation inhibition and causing the decrease in P-gp production (Dos Santos et al., 2010). In addition, IL-6 induced C/EBPs which triggered MRP2 transcription and led to the increase in MRP2 production (Ramji and Foka, 2002). Moreover, glucocorticoids binding with glucocorticoid receptor (GR) reacted with glucocorticoid response element (GRE) leading to silent *MDR1* gene transcription (Ramji and Foka, 2002). This might be explained why the stress from environment that might trigger the resistance mechanism in cTVT.

In the resistance cTVT, the *MDR1* mRNA expression (evaluated by qPCR) and P-gp expression (evaluated by IHC and WB) were lower in the resistance group than the untreated group in this study. These findings were opposite to the reports in the other tumors resistance mechanisms (Gramer et al., 2013; Pawlowski et al., 2013; Zandvliet et al., 2014). On the other hand, the

MRP2 mRNA expression seemed to be increased in the resistance group compared with the untreated group and the MRP2 expression was significant higher in IHC study. From this finding was similar to the expression of MRP2 in resistance human gall bladder carcinoma that resistance-associated protein expressed increasingly (Kim et al., 2013). From our results revealed the *MRP1* mRNA and protein expression did not differ between resistance and untreated groups. From the present study showed the differences among weeks of treatment and in the resistance cTVT suggesting that MRP2 might be involving in the resistance mechanism in cTVT rather than P-gp and MRP1 that has been discovered previously. This suggesting was supported by the study of Gerardi et al. (2014) that glutathione-s-transferase was found in the resistance cTVT which glutathione was used for drug co-transportation in MRPs. The expression of this enzyme showed the increase of glutathione utilization in cTVT cells that might result from the drug conjugation with glutathione for drug co-transportation by MRP2 that involved in the resistance mechanism of resistance cTVT. Then, the higher expression of MRP2 in this study supporting the finding of glutathione-s-transferase from the study of Gerardi et al. (2014) that glutathione was necessary for vincristine resistance mechanism.

In clinical application from this study, drug-resistance associated proteins found in cTVT might help the practitioner to select the appropriate drug for the resistance cTVT. In this study, the combination of L-asparaginase and vincristine could be treated the resistance. L-asparaginase is the enzyme that converts asparagine to aspartic acid and ammonia. From the study of Balasubramanian et al. (2013) suggested the restriction of asparagine synthetase activity in tumor cells. The using of L-asparaginase causes depletion of tumor cells' asparagine pool which is necessary for protein synthesis, leads to tumor regression (Capizzi et al., 1970; Muller and Boos, 1998; Balasubramanian et al., 2013). L-asparaginase was added into the common treatment protocol i.e. acute lymphoblastic leukemia or lymphoma in human and lymphoma in dog (Barton, 2001; Northrup et al., 2002; Narta et al., 2007). Then, L-asparaginase

was combined with vincristine for resistance cTVT treatment in this study due to L-asparaginase is an enzyme that was not degraded or excreted via MRP2 (Mealey et al., 1998; Gottesman et al., 2002). After treatment, all resistance cases achieved complete remission. This might be explained that L-asparaginase could suppress the tumor growth through the limiting of asparagine pool in the resistance cTVT cells and work together with vincristine to induce tumor regression. This finding was the strongly support of this study that MRP2 was the majority in the resistance mechanism of cTVT.

Conclusions

This study demonstrated that the dog affected with large-size and extra-genital cTVT are prone to be resistance to the vincristine therapy. Moreover, vincristine treatment during hot season likely compromised the dog immunity, contributing to the higher number of vincristine injections required for the complete remission. In cTVT, the inhibition of P-gp protein expression (IHC) is triggered by the vincristine treatment at week 3rd. The drug resistance mechanism related to ATP-binding cassettes (ABC) transporters was characterized by the decrease of P-gp mRNA and protein and the increase of MRP2 protein. Moreover, the clinical cases have proved that the L-asparaginase and vincristine combination is effective in the treatment of cTVT resistances. It is possible that L-asparaginase does not excrete through the ABC transporters including MRP2. Therefore, L-asparaginase is suggested as an alternative to other highly hepatotoxic anticancer drug i.e. doxorubicin and methotrexate, to be used in the vincristine resistance cTVT. The recently results are beneficial for the prediction of treatment outcome and selection of anti-cancer drug in vincristine resistance cTVT. However, further investigations to demonstrate all signal transduction of L-asparaginase will be of useful in the clinical practices.

REFERENCES

Balasubramanian MN, Butterworth EA and Kilberg MS 2013. Asparagine synthetase: regulation by cell stress and involvement in tumor biology. Am J Physiol Endocrinol Metab. 304(8): E789-799.

Baldini N, Scotlandi K, Barbanti-Brodano G, Manara MC, Maurici D, Bacci G, Bertoni F, Picci P, Sottili S, Campanacci M and et al. 1995. Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome. N Engl J Med. 333(21): 1380-1385.

Barton CL 2001. Chemotherapy. In: Small animal clinical pharmacology and therapeutics. D.M. Boothe (ed). Philadelphia, USA: W.B. Saunders. 331-348.

Bastan A, Acar DB and Cengiz M 2008. Uterine and ovarian metastasis of transmissible venereal tumor in a bitch. Turk J Vet Anim Sci. 32(1): 65-66.

Boscos C 1988. Transmissible venereal tumour in the dogs: clinical observations and treatment. Animalis Familiaris. 3: 10-15.

Boscos CM and Ververidis HN 2004. Canine TVT –Clinical findings, Diagnosis and Treatment. 29th World Small Animal Veterinary Association, Rhodes, Greece.

Brown NO, Calvert C and MacEwen EG 1980. Chemotherapeutic management of transmissible venereal tumors in 30 dogs. J Am Vet Med Assoc. 176(10 Pt 1): 983-986.

Calvert CA, Leifer CE and MacEwen EG 1982. Vincristine for treatment of transmissible venereal tumor in the dog. J Am Vet Med Assoc. 181(2): 163-164.

Capizzi RL, Bertino JR and Handschumacher RE 1970. L-asparaginase. Annu Rev Med. 21: 433-444.

Chan HS, Haddad G, Thorner PS, DeBoer G, Lin YP, Ondrusek N, Yeger H and Ling V 1991. P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. N Engl J Med. 325(23): 1608-1614.

Coppoc GL 2009. Chemotherapy of neoplastic diseases. In: Veterinary Pharmacology and Therapeutics. 9 ed. J.E. Riviere and M.G. Papich (eds). Iowa, USA: Willey-Balckwell. 1205-1231.

Das U and Das AK 2000. Review of canine transmissible venereal sarcoma. Vet Res Commun. 24(8): 545-556.

Dos Santos NR, Ghezzo MN, da Silva RC and Fernandes MT 2010. NF-kappaB in T-cell Acute Lymphoblastic Leukemia: Oncogenic Functions in Leukemic and in Microenvironmental Cells. Cancers (Basel). 2(4): 1838-1860.

Fernandez C, Buyse M, German-Fattal M and Gimenez F 2004. Influence of the pro-inflammatory cytokines on P-glycoprotein expression and functionality. J Pharm Pharm Sci. 7(3): 359-371.

Fonseca LS, Mota LSLS, Colodel MM, Ferreira I, Brandao CVS and Rocha NS 2012. Spontaneous canine transmissible venereal tumor: association between different phenotypes and the insertion LINE-1/c-myc. Rev Colomb Cienc Pec. 25(3): 402-408.

Ganguly B, Das U and Das AK 2013. Canine transmissible venereal tumour: a review. *in* Vet Comp Oncol.

Gaspar LFJ, Ferreira I, Colodel MM, Brandao CVS and Rocha NS 2010. Spontaneous canine transmissible venereal tumor: cell morphology and influence on P-glycoprotein expression. Turk J Vet Anim Sci. 34(5): 447-454.

Gerardi DG, Tinucci-Costa M, Silveira ACT and Moro JV 2014. Expression of P-glycoprotein, multidrug resistance-associated protein, glutathione-S-transferase pi and p53 in canine transmissible venereal tumor. Pesqui Vet Brasil. 34(1): 71-78.

Gillet JP, Efferth T and Remacle J 2007. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. Biochimica et Biophysica Acta. 1775(2): 237-262.

Ginn PE 1996. Immunohistochemical detection of P-glycoprotein in formalin-fixed and paraffin-embedded normal and neoplastic canine tissues. Vet Pathol. 33(5): 533-541.

Gonzalez CM, Griffey SM, Naydan DK, Flores E, Cepeda R, Cattaneo G and Madewell BR 2000. Canine transmissible venereal tumour: A morphological and immunohistochemical study of 11 tumours in growth phase and during regression after chemotherapy. J Comp Pathol. 122(4): 241-248.

Gottesman MM, Fojo T and Bates SE 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nature reviews Cancer. 2(1): 48-58.

Gramer I, Kessler M and Geyer J 2013. Determination of MDR1 gene expression for prediction of chemotherapy tolerance and treatment outcome in dogs with lymphoma. Vet Comp Oncol.

Hekman JP, Karas AZ and Sharp CR 2014. Psychogenic Stress in Hospitalized Dogs: Cross Species Comparisons, Implications for Health Care, and the Challenges of Evaluation. Animals (Basel). 4(2): 331-347.

Hicks TA, McGlone JJ, Whisnant CS, Kattesh HG and Norman RL 1998. Behavioral, endocrine, immune, and performance measures for pigs exposed to acute stress. J Anim Sci. 76(2): 474-483.

Hirose M 2002. Biology and modulation of multidrug resistance (MDR) in hematological malignancies. Int J Hematol. 76 Suppl 2: 206-211.

Honscha KU, Schirmer A, Reischauer A, Schoon HA, Einspanier A and Gabel G 2009. Expression of ABC-transport proteins in canine mammary cancer: consequences for chemotherapy. Reprod Domest Anim. 44 Suppl 2: 218-223.

Hsiao YW, Liao KW, Chung TF, Liu CH, Hsu CD and Chu RM 2008. Interactions of host IL-6 and IFN-gamma and cancer-derived TGF-beta1 on MHC molecule expression during tumor spontaneous regression. Cancer Immunol Immunother. 57(7): 1091-1104.

Hsiao YW, Liao KW, Hung SW and Chu RM 2002. Effect of tumor infiltrating lymphocytes on the expression of MHC molecules in canine transmissible venereal tumor cells. Vet Immunol Immunop. 87(1-2): 19-27.

Hsiao YW, Liao KW, Hung SW and Chu RM 2004. Tumor-infiltrating lymphocyte secretion of IL-6 antagonizes tumor-derived TGF-beta 1 and restores the lymphokine-activated killing activity. J Immunol. 172(3): 1508-1514.

Kelly CF and Bond TE 1971. Bioclimatic factors and their measurement. In: A Guide to Environmental Research on Animals. R.G. Yeck, R.E. McDowell, T.E. Bond, R.W. Dougherty, T.E. Hazen, H.D. Johnson, J.E. Johnston, C.F. Kelly, N. Pace, S.Y. Smith, L.C. Ulberg, and W.O. Wilson (eds). Washington DC: National Academy of Science Press. 7-93.

Kim HS, Kim NC, Chae KH, Kim G, Park WS, Park YK and Kim YW 2013. Expression of multidrug resistance-associated protein 2 in human gallbladder carcinoma. Biomed Res Int. 2013: 527534.

Kunakornsawat S, Imsilp K, Yatbanthoong N, Ratanapob N, Supsavhad W, Sreesampan S and Nuklang G 2009. Vincristine chemotherapeutic treatment for transmissible venereal tumor in 60 dogs. KPS. 8(2): 79-91.

Kunakornsawat S, Yippaditr W, Jamjan N, Bootcah R, Netramai S, Viriyarumpa J and Kornkaewrat K 2010. Surgical correction of transmissible venereal tumor with vincristine-resistance using episiotomy and vulvovaginoplasty in female and subtotal penile amputation and scrotal ablation in male dogs. 48th Kasetsart University Annual Conference: Veterinary Medicine, Bangkok:191-200.

Lacetera N, Bernabucci U, Scalia D, Ronchi B, Kuzminsky G and Nardone A 2005. Lymphocyte functions in dairy cows in hot environment. Int J Biometeorol. 50(2): 105-110.

Lee JY, Tanabe S, Shimohira H, Kobayashi Y, Oomachi T, Azuma S, Ogihara K and Inokuma H 2007. Expression of cyclooxygenase-2, P-glycoprotein and multi-drug resistance-associated protein in canine transitional cell carcinoma. Res Vet Sci. 83(2): 210-216.

Linharattanaruksa P, Chatdarong K, Ponglowhapan S, Khalid M and Srisuwatanasagul S 2013a. Immunolocalization of Prostaglandin E2 Receptor Subtype 4 (EP4) in the Cervix of Cyclic Bitches and Those with Pyometra. Pak Vet J. 33(3): 340-345.

Linharattanaruksa P, Swangchan-Uthai T, Srisuwatanasagul S, Khalid M and Chatdarong K 2013b. mRNA and Protein Expressions of Prostaglandin E-2 Receptors (EP2 and EP4), Cyclooxygenase-2 and Prostaglandin E Synthase in

the Cervix of Cyclic Bitches and those with Pyometra. Thai J Vet Med. 43(2): 219-227.

Lorimier LP and Fan TM 2001. Canine Transmissible Venereal Tumor. Pages 799-802 *in* WITHROW and MACEWAN's Small Animal Clinical Oncology. 4th ed. S.J. Withrow and D.M. Vail, eds. WB Saunders, Philadelphia, USA.

Materna V, Pleger J, Hoffmann U and Lage H 2004. RNA expression of MDR1/P-glycoprotein, DNA-topoisomerase I, and MRP2 in ovarian carcinoma patients: correlation with chemotherapeutic response. Gynecol Oncol. 94(1): 152-160.

Mealey KL, Barhoumi R, Rogers K and Kochevar DT 1998. Doxorubicin induced expression of P-glycoprotein in a canine osteosarcoma cell line. Cancer Lett. 126(2): 187-192.

Medary I, Aronson D, Cheung NKV, Ghavimi F, Gerald W and LaQuaglia MP 1996. Kinetics of primary tumor regression with chemotherapy: Implications for the timing of surgery. Ann Surg Oncol. 3(6): 521-525.

Mello Martins MI, Ferreira de Souza F and Gobello C 2005. The canine transmissible venereal tumor: etiology, pathology, diagnosis and treatment. *in* Recent Advances in Small Animal Reproduction. Concannon P.W., England G., Veretgegen J., and Linde-Forsberg C., eds. International Veterinary Information Service, Ithaca, New York.

Mozos E, Mendez A, GomezVillamandos JC, delasMulas JM and Perez J 1996. Immunohistochemical characterization of canine transmissible venereal tumor. Vet Pathol. 33(3): 257-263.

Muller HJ and Boos J 1998. Use of L-asparaginase in childhood ALL. Crit Rev Oncol Hematol. 28(2): 97-113.

Murgia C, Pritchard JK, Kim SY, Fassati A and Weiss RA 2006. Clonal origin and evolution of a transmissible cancer. The Cell. 126(3): 477-487.

Nakaichi M, Takeshita Y, Okuda M, Nakamoto Y, Itamoto K, Une S, Sasaki N, Kadosawa T, Takahashi T and Taura Y 2007. Expression of the MDR1 gene and P-glycoprotein in canine mast cell tumor cell lines. J Vet Med Sci. 69(2): 111-115.

Narta UK, Kanwar SS and Azmi W 2007. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. Crit Rev Oncol Hematol. 61(3): 208-221.

Noma B, Sasaki T, Fujimoto Y, Serikawa M, Kobayashi K, Inoue M, Itsuki H, Kamigaki M, Minami T and Chayama K 2008. Expression of multidrug resistance-associated protein 2 is involved in chemotherapy resistance in human pancreatic cancer. Int J Oncol. 33(6): 1187-1194.

Northrup NC, Rassnick KM, Snyder LA, Stone MS, Kristal O, Cotter SM and Moore AS 2002. Neutropenia associated with vincristine and L-asparaginase induction chemotherapy for canine lymphoma. J Vet Intern Med. 16(5): 570-575.

Osipov NE and Golubeva VA 1976. [Diagnosis and treatment of transmissible sarcoma]. Veterinaria. (7): 97-98.

Otomo K, Koike T, Kudo T and Sakai T 1981. Histological and Ultrastructural findings of regressing canine transmissible venereal tumor after repeated transplantation. Nihon juigaku zasshi. 43(6): 823-832.

Pawlowski KM, Mucha J, Majchrzak K, Motyl T and Krol M 2013. Expression and role of PGP, BCRP, MRP1 and MRP3 in multidrug resistance of canine mammary cancer cells. BMC Vet Res. 9: 119.

Petterino C, Rossetti E, Bertoncello D, Martini M, Zappulli V, Bargelloni L and Castagnaro M 2006. Immunohistochemical detection of P-glycoprotein (clone C494) in canine mammary gland tumours. J Vet Med A Physiol Pathol Clin Med. 53(4): 174-178.

Ramji DP and Foka P 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J. 365(Pt 3): 561-575.

Rogers KS 1997. Transmissible Venereal Tumor. Compend Contin Educ Vet. 19(9): 1036-1045.

Rogers KS, Walker MA and Dillon HB 1998. Transmissible venereal tumor: a retrospective study of 29 cases. J Am Anim Hosp Assoc. 34(6): 463-470.

Rumjanek VM, Trindade GS, Wagner-souza K, Meletti-DE-Oliveira MC, Marques-Santos LF, Maia RC and Capella MAM 2001. Multidrug resistance in tumor cells:

characterization of the multiresistance cell line K562-Lucena1. An Acad Bras. 73(1): 57-69.

Said RA, Silva LF, Albuquerque AROL, Sousa-Neta EM and Lavinsky MO 2009. Efficacy and side effects of vincristine sulfate treatment on canine transmissible venereal tumor. the 34th World Small Animal Veterinary Association, São Paulo, Brazil.

Santos de Amaral A, Bassani-Silva S, Ferreira I, Fonseca LS, Andrade FHE, Gasper LFJ and Rocha NS 2007. Cytomorphological characterization of transmissible venereal tumor. Rev Port Ciênc Vet. 102(563-564): 253-260.

Scarpelli KC, Valladao ML and Metze K 2010. Predictive factors for the regression of canine transmissible venereal tumor during vincristine therapy. Vet J. 183(3): 362-363.

Stockmann D, Ferrari HF, Andrade AL, Cardoso TC and Luvizotto MCR 2011. Detection of the tumour suppressor gene TP53 and expression of p53, Bcl-2 and p63 proteins in canine transmissible venereal tumour. Vet Comp Oncol. 9(4): 251-259.

Sukhai M, Yong A, Pak A and Piquette-Miller M 2001. Decreased expression of P-glycoprotein in interleukin-1beta and interleukin-6 treated rat hepatocytes. Inflamm Res. 50(7): 362-370.

Sulova Z, Mislovicova D, Gibalova L, Vajcnerova Z, Polakova E, Uhrik B, Tylkova L, Kovarova A, Sedlak J and Breier A 2009. Vincristine-induced overexpression of P-glycoprotein in L1210 cells is associated with remodeling of cell surface saccharides. J Proteome Res. 8(2): 513-520.

Swangchan-Uthai T, Walsh SW, Alexander SL, Cheng Z, Crowe MA, Evans AC and Wathes DC 2011. Comparison of mRNA for IGFs and their binding proteins in the oviduct during the peri-oestrous period between dairy heifers and lactating cows. Reproduction. 142(3): 457-465.

Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C and Gottesman MM 2006. Targeting multidrug resistance in cancer. Nat Rev Drug Discov. 5(3): 219-234.

Tada Y, Wada M, Migita T, Nagayama J, Hinoshita E, Mochida Y, Maehara Y, Tsuneyoshi M, Kuwano M and Naito S 2002. Increased expression of multidrug

resistance-associated proteins in bladder cancer during clinical course and drug resistance to doxorubicin. Int J Cancer. 98(4): 630-635.

Theewasutrakul P, Manachai N, Wangnaitham S and Rungsipipat A 2007. Successful treatment of canine cutaneous lymphoma by combination L-asparaginase and vincristine sulfate. 35th Thai Veterinary Medical Association, Bangkok, Thailand:43-46.

Tuntivanich P 1983. Chemotherapeutics treating transmissible venereal tumor (TVT) in dogs. Thai J Vet Med. 13(2): 104-121.

Uozurmi K, Nakaichi M, Yamamoto Y, Une S and Taura Y 2005. Development of multidrug resistance in a canine lymphoma cell line. Res Vet Sci. 78(3): 217-224.

Yamaguchi S, Zhao YL, Nadai M, Yoshizumi H, Cen X, Torita S, Takagi K, Takagi K and Hasegawa T 2006. Involvement of the drug transporters p glycoprotein and multidrug resistance-associated protein Mrp2 in telithromycin transport. Antimicrob Agents Chemother. 50(1): 80-87.

Yang YX, Xiao ZQ, Chen ZC, Zhang GY, Yi H, Zhang PF, Li JL and Zhu G 2006. Proteome analysis of multidrug resistance in vincristine-resistant human gastric cancer cell line SGC7901/VCR. Proteomics. 6(6): 2009-2021.

Zandvliet M, Teske E and Schrickx JA 2014. Multi-drug resistance in a canine lymphoid cell line due to increased P-glycoprotein expression, a potential model for drug-resistant canine lymphoma. Toxicol In Vitro. 28(8): 1498-1506.

APPENDIX

Appendix A

List of publication and conference proceedings

1. Sudjaidee, P, Ponglowhaphan, S, Tummaruk, P and Chatdarong, K 2015. Factor affecting treatment outcome of canine transmissible venereal tumor: a retrospective study. **Thai J Vet Med** 45(4) (accepted)
2. Sudjaidee, P, Theewasutrakul, P, Techarungchaikul, S, Ponglowhaphan, S and Chatdarong, K 2012. Treatment of canine transmissible venereal tumor using vincristine sulfate combined with L-asparaginase in clinical vincristine-resistant cases: a case report. **Thai J Vet Med** 42(1): 117-122.
3. Sudjaidee, P, Theewasutrakul, P, Techarungchaikul, S, Ponglowhaphan, S and Chatdarong, K, 2011. Treatment of vincristine resistant canine transmissible venereal tumor by combining of L-asparaginase and vincristine. **The 1st symposium of the Thai society for animal reproduction** 29-30 August 2011.
4. Sudjaidee, P, Srisuwatanasagul, S, Ponglowhaphan, S and Chatdarong, K 2014. Expression of multidrug resistance associated proteins in canine transmissible venereal tumor during vincristine sulfate treatment. **The 2st symposium of the Thai society for animal reproduction.** 20-21 March 2014.
5. Sudjaidee, P, Sawangchan-uthai, T, Srisuwatanasagul, S, Ponglowhaphan, S and Chatdarong, K 2015. Multidrug resistance associated gene and protein expression in resistant canine transmissible venereal tumor. **The 14th Chulalongkorn University Veterinary Conference.** 20-22 April 2015

VITA

Mr. Pansawut Sudjaidee was born on July 20th 1984 in Chonburi province, Thailand. He graduated with Degree of Doctor of Veterinary Medicine (DVM) with the 2nd honour from Faculty of Veterinary Science, Mahidol University, in 2007. In 2009, he received the scholarship from the Faculty Development Scholarship from Commission on Higher Education to perform a PhD program of Theriogenology at Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. His focus research is about the mechanism of drug resistance in canine transmissible venereal tumor related to multidrug resistance associated protein expression during treatment, which aims to find out the association of treatment outcome, multidrug resistance associated gene and protein expression and vincristine resistance.