DETECTION ON THE EXPRESSION OF THE TRANSCRIPTION FACTORS: *OCT-4*, *NANOG*, AND *SOX-2* GENES IN CANINE CUTANEOUS MAST CELL TUMORS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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การตรวจการแสดงออกของยีนทรานสคริปชันแฟคเตอร์ชนิด *Oct-4 Nanog* และ *Sox-2* ในเนื้องอกผิวหนังสุนัขชนิดมาสต์เซลล์

น.ส.ศิริลักษณ์ มีสุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ศิริลักษณ์ มีสุวรรณ : การตรวจการแสดงออกของยีนทรานสคริปชันแฟคเตอร์ชนิด *Oct-4 Nanog* และ *Sox-2* ในเนื้องอกผิวหนังสุนัขชนิดมาสต์เซลล์. (DETECTION ON THE EXPRESSION OF THE TRANSCRIPTION FACTORS: *OCT-4*, *NANOG*, AND *SOX-2* GENES IN CANINE CUTANEOUS MAST CELL TUMORS) อ.ที่ปรึกษาหลัก : ศ. ดร.อัจฉริยา ไศละสูตD.V.M., FRVCS, Ph.D., DTBVP, อ.ที่ปรึกษาร่วม : ผศ. ดร.ประพฤติดี ปิยะวิริยะกุลD.V.M., M.Sc., Ph.D.,อ. ดร. เกษม รัตนภิญโญพิทักษ์D.V.M., M.Sc., Ph.D., DTBVP

งานวิจัยนี้มีวัตถุประสงค์เพื่อตรวจการแสดงออกของทรานสคริปชันแฟคเตอร์ Oct-4, Nanog และ Sox-2 ทั้งในระดับยีน และโปรตีน ในตัวอย่างเนื้องอกที่ผิวหนังสุนัขชนิดมาสต์เซลล์จำนวน 30 ตัว ที่เข้ามารับการรักษาที่โรงพยาบาลสัตว์เล็ก คณะ สัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย แบ่งตัวอย่างออกเป็น 2 ส่วน ส่วนแรกนำไปตรวจทางจุลพยาธิวิทยาโดย ย้อมสี H&E เพื่อจำแนกเกรดของเนื้องอก โดยใช้วิธีการจจำแนกเป็น 2 ระบบ คือ Patnaik.s system แบ่งออกได้เป็น 3 เกรด คือ เกรด I II และ III พบว่าตัวอย่างเนื้องอกจำแนกเป็น เกรด I, 40% (12/30) เกรด II, 26.67% (8/30) และ เกรด III, 33.33% (10/30) และ Kiupel's system แบ่งเป็น 2 เกรด คือ เกรดต่ำ และ เกรดสูง พบว่ามี เกรดต่ำ 66.67% (20/30) และ เกรดสูง 33.33% (10/30) ตามลำดับ ตรวจการแสดงออกของ Oct-4, Nanog และ Sox-2 ยีน ใช้เทคนิค RT-PCR และ qRT-PCR พบว่าพบการแสดงออกของ Oct-4 และ Sox-2, (100%; 30/30) ส่วน Nanog ไม่สามารถตรวจพบการแสดงออกของยีนได้ (0%) ส่วนเทคนิค qRT-PCR พบระดับการแสดงของ Oct-4 ในเกรด III และ เกรดสูง สูงกว่าในเกรด I II และ เกรดต่ำ ตามลำดับ ซึ่งผลที่ได้ตรงข้ามกับระดับการแสดงออกของ Sox-2 ใน เกรด III และ เกรดสูง มีแนวโน้มต่ำกว่าในเกรด I, II และ เกรดต่ำตามลำดับ เมื่อเปรียบเทียบกับยีนควบคุม ตรวจการแสดงออกทางอิมมูโนฮีสโตเคมีของ Oct-4, Nanog และ Sox-2 พบตัวอย่างที่มีการแสดงออกของ Oct-4, 90% (27/30); Nanog, 80% (24/30) และ Sox-2 80% (24/30) ตามล าดับ ผล วิเคราะห์จ านวนเซลล์ที่ให้ผลบวกกับการแสดงออกของ Oct-4, Nanog และ Sox-2 พบว่าจำนวนเซลล์ที่ให้ผลบวกกับการ แสดงออกของ Oct-4 และ Nanog ไม่มีความแตกต่างกันอย่างมีนัยสำคัญในทุกเกรด ส่วน การแสดงออกของ Sox-2 มีความ แตกต่างอย่างมีนัยสำคัญทางสถิติ ($p \leq 0.05$) การตรวจการแสดงออกของโปรตีน Oct-4, Nanog และ Sox-2 โดยเทคนิค Western blot พบแถบปฏิกิริยาการแสดงออกของ Oct-4 จำนวน 1 แถบที่ 43 kDa, Nanog จำนวน 3 แถบ โดยประมาณ ที่ 45,35 และ 25 kDa และ Sox-2 จำนวน 4 แถบ โดยประมาณที่ 77,65,35 และ 26 kDa ตามลำดับ โดยแถบปฏิกิริยาที่ ได้จะมีสัญญาณระดับอ่อนและไม่ชัดเจน เมื่อเทียบกับตัวควบคุมบวก นอกจากนี้ได้ตรวจการแสดงออกของโปรตีน Oct-4 Nanog และ Sox-2 ด้วยเทคนิค Intranuclear Immunocytofluorescence พบว่าเทคนิคนี้สามารถตรวจบริเวณการ แสดงออกของโปรตีน Oct-4, Nanog และ Sox-2 ภายในนิวเคลียส และไซโตพลาสมของเซลล์มะเร็ง นอกจากนี้งานวิจัยนี้ได้ ใช้เทคนิค Surface Plasmon resonance (SPR) พบว่ามีศักยภาพในการตรวจการแสดงออกของโปรตีน Oct-4, Nanog และ Sox-2 ในตัวอย่างเนื้องอกได้ อย่างไรก็ตามเทคนิคนี้มีความไวค่อนข้างสูง และยังมีข้อจำกัดทางเทคนิคในความจำเพาะซึ่งควรมีการพัฒนาต่อไป จากผลงานวิจัยนี้พบว่าสามารถตรวจพบการแสดงออกของทรานสคริปชันแฟคเตอร์ Oct-4 Nanog และ Sox-2 ทั้งในระดับยีนและโปรตีนในเนื้องอกผิวหนังสุนัขชนิดมาสต์เซลล์ซึ่งน่าจะเป็นประโยชน์ในการพัฒนาเป็น ตัวบ่งชี้ความรุนแรงของมะเร็งต่อไป

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Sirilak Meesuwan : DETECTION ON THE EXPRESSION OF THE TRANSCRIPTION FACTORS: *OCT-4*, *NANOG*, AND *SOX-2* GENES IN CANINE CUTANEOUS MAST CELL TUMORS. Advisor: Prof. Dr. ACHARIYA SAILASUTA, D.V.M., FRVCS, Ph.D., DTBVP Co-advisor: Asst. Prof. Dr. PRAPRUDDEE PIYAVIRIYAKUL, D.V.M., M.Sc., Ph.D.,Dr. KASEM RATTANAPINYOPITUK, D.V.M., M.Sc., Ph.D., DTBVP

The objectives of this study were to determine the expression of Oct-4, Nanog, and Sox-2 in both mRNA and proteins levels. The 30 biopsied MCT cases, from the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, were used in this study. The samples were divided into two parts; part I, the biopsied samples were processed for paraffin section and stained with H&E stain, for grading based on Patnaik's system and Kiupel's system and the other part was detected the expression of Oct-4, Nanog, and Sox-2 genes. The MCT samples were classified into three grades; grade I, II, and III by Patnaik system; grade I, 40% (12/30), grade II, 26% (8/30) and grade III, 33.33% (10/30). And, Kiupel's system which were low grade, 66.66% (20/30) and high grade, 33.33% (10/30) respectively. The RT-PCR and qRT-PCR were used for detecting Oct-4, Nanog, and Sox-2 mRNA in MCT cases. The results showed that all of MCT samples expressed both Oct-4 and Sox-2 at mRNA level (100 %; 30/30). While, Nanog could not be detected in all MCT samples (0%). For qRT-PCR, the Oct-4 gene in high grade tended to be higher than low grade parallelly in grade III also tended to be higher than grade I and II respectively . On the contrary, Sox-2 expression, in grade III and high grade tended to be lower than grade I and low grade respectively, when compared to internal control. However, the results had no statistically significant for the experiments among the grading system. The immunohistochemical expression of Oct-4, Nanog, and Sox-2 in MCT samples were conducted. There were positive expression of Oct-4, Nanog, and Sox-2 in all the samples at 90% (27/30), 80 % (24/30) and 80% (24/30) respectively. Regards to the positive cells and the grade of MCT, it was demonstrated that there were no statistically difference of Oct-4 and Nanog positive cells in all grades ($p \geq 0.05$) . On the contrary, the positive cells of Sox-2 in high grade were higher than low grade MCT samples with statistically difference ($p \leq 0.05$). The results of Western blot technique showed the expression of Oct-4, Nanog, and Sox-2 protein with low reaction intensity. Which were; Oct-4, 1 band at 43 kDa, Nanog, 3 bands, at 45,35 and 25 kDa, approximately, Sox-2, 4 bands, at 77,65,35 and 26 kDa approximately in all MCT samples respectively when compared to positive control.. In addition, the demonstration of Intranuclear Immunocytofluorescence was conducted on the expression of Oct-4, Nanog, and Sox-2 proteins which the positively localizations were shown in intranuclear and intracytoplasmic of MCT cells. Additionally, the expression of the Oct-4, Nanog, and Sox-2 proteins could be potentially detected by Surface Plasmon Resonance, SPR technique in MCT. However, there were some limitations of the technique, due to their high sensitivity and nonspecific appearances. By the obtained results, it is revealed that the major embryonic transcription factors, Oct-4, Nanog, and Sox-2 mRNA and proteins in the MCT tissue and could be detected. Which could be possibly beneficial on the development of the prognostic marker of this tumor in the future.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Sirilak Meesuwan

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LIST OF ABBREVIATIONS

μl	microliter
μm	micrometer
CSC	Cancer s cell
DAB	diaminobenzidine tertrahydrochoride
ESC	Embryonic stem cell
hrs	hours
IHC	Immunohistochemistry
IF	Immunocytofluorescence
min	minute
mRNA	messenger ribonucleic acid
MPOH	3-mercaptopropanol
MUA	mercaptoundecanoic acid
Oct-4	Octamer-binding transcription factor in the POU
PBS	Phosphate buffer saline
PE	Phycoerythrin
PMSF	phenylmethysulfonyl fluoride
PVDF	polyvinylidene difluoride nitrocellulose membrane
RT-PCR	Reverse transcription polymerase chain reaction

LIST OF ABBREVIATIONS

qRT-PCR	quantitative Reverse transcription polymerase chain reaction
SAM	self-assembled preparation
second	sec
SPR	Surface Plasmon Resonance
TBST	Tris-buffered saline

CHAPTER I

INTRODUCTION

1. Background and rationale

Canine cutaneous mast cell tumor (MCT) is a haematopoietic disorders in dogs that composed of neoplastic mast cell. MCT is the second most of skin tumor malignancy and its is relatively accounted around 27% (Rungsipipat et al., 2003; Kasorndorkbua, 2008; Welle et al., 2008). The biological behaviors of MCT appearance on recurrence and difficult to treatment. Previous studies suggested that cancers which contained Cancer stem cells (CSCs) usually recurred and metastasis after treatment. Moreover, MCT has been reported that composed of putative cancer stem cells based on OCT4-immunohistochemistry (IHC) (Webster et al., 2007b). Interestingly in medicine, many studies have reported putative cancer stem cells in various tumors have high. For examples, CSCs were found in various cancers including hematopoietic, head and neck, prostate, lung, brain, colon, skin, liver and pancreatic cancers (Song et al., 2013; Kreso and Dick, 2014; Oskarsson et al., 2014).

Cancer stem cells (CSC) are the typical group of neoplastic cells capable to replenish all neoplastic cell subpopulations in a given tumor and this biological proprietary is fundamentally referred to self-renewal. Moreover, they are considered being immortal and responsible for disease propagation, recurrence, metastasis and chemotherapeutic resistance (Nguyen et al., 2012; Oskarsson et al., 2014; Plaks et al., 2015). The markers of embryonic stem cells or normal stem cell self- renewal and pluripotency properties are the transcription factors (Oct- 4, Nanog, Sox-2, c-Myc and Klf). Especially, the three major transcription factors, Oct-4, Nanog and Sox-2 have been detected CSCs in many cancers such as lung, prostate, pancreatic and breast cancer

(Dong et al., 2004; Tai et al., 2005; Schulenburg et al., 2006; Dalerba et al., 2007; Ferletta et al., 2011; Leis et al., 2012; Amaya and Bryan, 2015; Wilson-Robles et al., 2015).

In veterinary science, there are a few reports on Oct-4 expression in putative CSCs in many cancers such as mast cell tumor, canine hepatocellular carcinomas, seminomas and thyroid carcinoma by immunohistochemistry (Webster et al., 2007b; Vargas et al., 2015) . In addition, the previous study and our preliminary study, demonstrated the Oct-4 expression at protein and mRNA levels by Immunohistochemistry and PCR technique, respectively (Eksiritrirat et al., 2012; Ketpun et al., 2013; Sailasuta et al., 2013). Hence, we believe that Oct-4 expressing cells might involve in the pathogenesis of MCT and MCT might have the stemness property. To ensure the research question is whether there are putative CSCs in MCT, we have hypothesized that the transcription factors; Oct-4, Nanog and Sox-2 should be express in MCT's cell population.

In this study, the determination on the self-renewal and pluripotency properties of putative CSCs in MCTs of the three major transcription factors; Oct-4, Nanog and Sox-2 expression will be detected in both mRNA and protein levels. RT-PCR and qRT-PCR. The methods that used to detect Oct-4, Nanog and Sox-2 proteins are immunohistochemistry, and western blotting technique. In addition, Intranuclear Immunocytofluorescence and Surface Plasmon Resonance (SPR) technique will be developed to analyse protein level with IHC and Western Blot parallel.

2. Studies of the thesis

In this study was determined on the self-renewal and pluripotency properties of putative CSCs in MCTs by the detection of the three major transcription factors; Oct-4, Nanog and Sox-2 expression.

3. Objectives

The objectives of this study are to:

1) To detect the expression of transcription factors; Oct-4, Nanog and Sox-2 genes at mRNA level by RT-PCR and qRT-PCR.

2) To detect the expression of transcription factors; Oct-4, Nanog and Sox-2 at protein level by Immunohistochemistry and Intranuclear Immunocytofluorescence.

3) To detect the level of transcription factors; Oct-4, Nanog and Sox-2 proteins by Western blot and the potential of Surface Plasmon Resonance (SPR) technique on detect the protein level of transcription factors; Oct-4, Nanog and Sox-2 proteins.

Keywords (Thai) เซลล์ต้นกำเนิด เนื้องอกผิวหนังสุนัขชนิดมาสต์เซลล์ อ็อกโฟร์ นาน็อก ซ็อกทู ยีนทรานสคริปชันแฟคเตอร์

Keywords (English) cancer stem cell, canine cutaneous mast cell tumor *Oct-4 Nanog, Sox-2* transcription factor gene

4. Research question

Does the canine cutaneous mast cell tumors (MCT) expressed self-renewal and pluripotency embryonic transcription factors?

5. Hypothesis

The detection of the expression of transcription factors; Oct-4, Nanog and Sox-2 genes and proteins can be demonstrated the stemness property of MCT tissues.

CHAPTET II

LITERATURE REVIEW

1.Canine cutaneous mast cell tumor (MCT)

Canine cutaneous mast cell tumor (MCT) is a tumor of heamatopoietic disorder which proliferation of neoplastic mast cell. MCT is a common skin tumor in the dogs and the affected dogs with almost 27% of malignancies (Rungsipipat et al., 2003; Kasorndorkbua, 2008; Welle et al., 2008). There are reported that boxer, boston terrier, bull terrier, and labrador retriever breed have high incidence rate of MCT more than other breeds. There is no relationship between sex and risk of MCT (Welle et al., 2008; Leidinger et al., 2014).

Generally, the normal mast cells derive from the myeloid stem cell and then a few mast cells migrate and mature in various tissues such as skin, intestinal tract and respiratory tract. The number of normal mast cells increased in the body during the immune process and then released proteins from many granules such as vasoactive amines, histamine, heparin, and proteolytic enzymes that can be effects on many body functions such as heart rate, blood pressure, and abnormalities in coagulation (Welle et al., 2008). In case of MCTs, mast cells can produce high level of degranulation product which effect of Paraneoplastic syndromes such as vomiting, duodenal ulcers, blood in the stool, abnormalities in coagulation, vasodilatation, and anaphylactic shock (Welle et al., 2008). Moreover, there have many reports showed that biological behaviors of MCTs can vary from slowly growing to rapid growing and invasion and can be found in dermis and sub-cutaneous of many tissues such as upper lip, trunk, head, neck, fore and hind limb (Welle et al., 2008).

2.Diagnosis

Diagnostic tool of MCT can be used fine needle aspiration (FNA) cytology but FNA can be not identified the grade of MCT. The biopsy is usually classified MCT grading to diagnostic and prognosis. The MCT cells are round to ovoid with 10 to 20 micrometers in diameter in case of well differentiated tumors and 12 to 35 micrometers in anaplastic tumors. Nuclei are round with or without nuclei. The cytoplasm of cells is filled with metachromatic granules (blue to purple).

The MCTs grading was first described by Bostock (Bostock, 1973). They are graded based on histopathological features of neoplastic cells. This system is three- tier grading; well, intermediately and poorly-differentiated grading. In well-differentiated grading, cytoplasms were clearly defined with regular, spherical, or oval nuclei. The mitotic figure is rarely seen. There are many largely intracytoplasmic granules. For intermediately - differentiated grading, there are dense cytoplasm with increased nucleus-to-cytoplamic ratio and mitotic figures and the poorly-differentiated grading, nuclei become to irregular in size and shape. The number of mitotic figures were increased while the number of cytoplasmic granules were decreased. After that, histologic grading used the criteria of Patnaik's system which are included cellular morphology, mitotic index, cellularity, extent of tissue involvement and the stromal reaction (Patnaik et al., 1984) which were divided into three grades, I, II and III (Fig.1). Grade I appeared in dermis with well differentiated cells, no evidence mitotic figures. In grade II, MCTs that contained in the deep dermis and subcutis has pleomorphism with mitotic figure 0-2/10 high power fields (HPF). Grade III, MCTs contained highly pleomorphic cells into dermal and subcutaneous. The large number of mitotic figures are seen (at least 3-6/10 HPF).

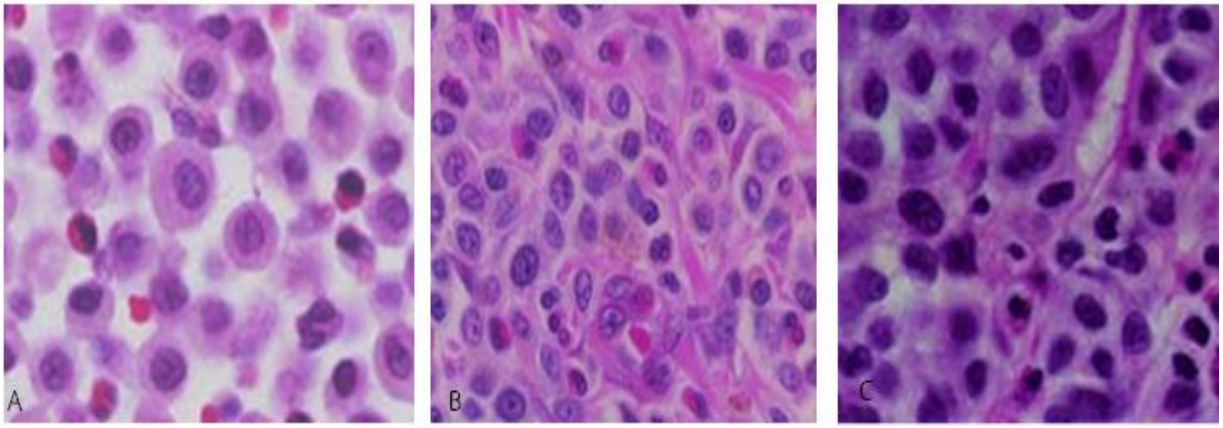
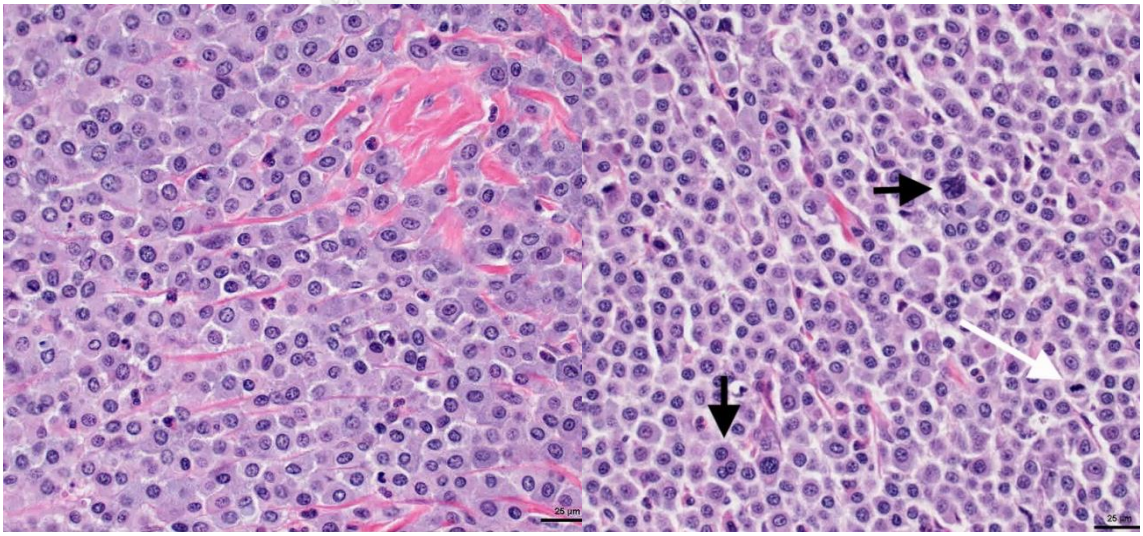


Figure 1. Histologic characteristics of cutaneous mast cell tumors in dogs.

Histologic characteristics of cutaneous mast cell tumors in dogs according to the Kiupel system . a low-grade cutaneous mast cell tumor on the left and a high-grade on the right. Note the uniform appearance of mast cells in the left-hand panel, as compared to the variability apparent on the right. The vertical and horizontal black arrows depict multinucleated and karyomegalic cells, respectively, while the white arrows illustrate a mitotic figures. HE stain, Bar=25 μm. (https://www.mspca.org/angell_services/tumor-grading-is-it-applicable/)



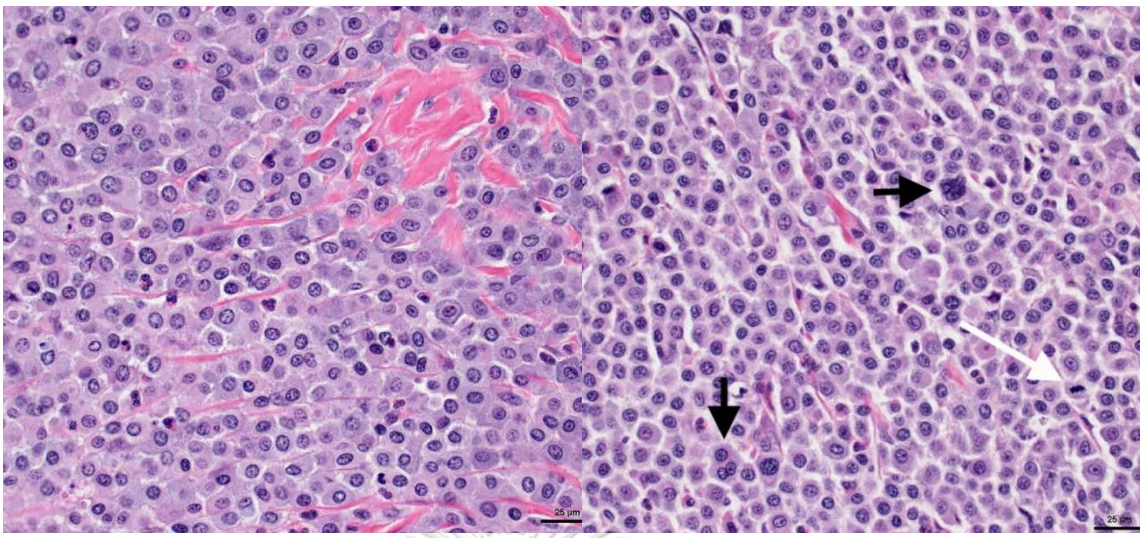


Figure 2. Histologic characteristics of cutaneous mast cell tumors in dogs.

Histologic characteristics of cutaneous mast cell tumors in dogs according to the Kiupel system . a low-grade cutaneous mast cell tumor on the left and a high-grade on the right. Note the uniform appearance of mast cells in the left-hand panel, as compared to the variability apparent on the right. The vertical and horizontal black arrows depict multinucleated and karyomegalic cells, respectively, while the white arrows illustrate a mitotic figures. HE stain, Bar=25 μm. (https://www.mspca.org/angell_services/tumor-grading-is-it-applicable/)

However, there are some biases from intra and interobserver and intratumoral heterogeneity from Both Bostock and Patnaik grading systems (Northrup et al., 2005). Recently, the two – tier grading system has been introduced. This system composed of low grade and high grade by using criteria of the mitotic rate and nuclear features (karyomegaly, multinucleated cells, and bizarre).The high grade included one of these criteria; a tumor with seven or greater mitotic figures per 10 HPF, three or greater multinucleated cells per 10 HPF, three or greater bizarre nuclei in 10 HPF and karyomegaly characterized by 10% of cells exhibiting nuclei varying by at least twice normal size (Kiupel et al., 2011).

In addition, Immunohistochemistry pattern of c-Kit has been employed for the progression of MCT (Sailasuta et al., 2014). The c-Kit (CD117) is a proto- oncogene that encode

the receptor tyrosine kinase. It has three portions; an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic kinase domain (Webster et al., 2006). The ligand of c-Kit binds to stem cell factor and then form a dimers of cytoplasmic kinase domain follow by activate phosphorylation. Activity of phospholylate is a signal for cell proliferation, growth and differentiation (Webster et al., 2006). The receptor tyrosine kinase Kit is a cell surface marker for several cells such as mast cells, hematopoietic progenitor cells, and germ cells (Webster et al., 2006). In a normal functional cells, stem cell factor stimulated mast cell growth and differentiated by bind with kit receptor and then activated receptor through phosphorylation.

Aberrations of *c-Kit* consists of mutations, deletions, and duplications, the mutation in exon11 (the juxtamembrane domain; cytoplasmic kinase domain) of *c-Kit* is leading to cancer by the kit receptor will be continuously activated leading to abnormality of cell proliferation (Webster et al., 2006).

3.Pathogenesis

The pathogenesis of MCTs may be unclear. The MCTs have been usually associated with chronic inflammation or skin irritants**.** In many studies revealed that genetic alterations introduced the risk of tumors. Especially, *c-Kit* mutations at exon11 (the juxtamembrane domain; cytoplasmic kinase domain) can be activated proliferation in the absence of growth factors leading to unlimited hyperproliferation of the cells (Ketpun et al., 2013; Sailasuta et al., 2014).

4.Treatment

Surgery and systemic chemotherapy are the treatments of choice for MCT treatment. However, the dogs with MCT still have the recurrence and metastasis after treatment. In surgical excision, surgical margins for invasive should be completely excised at least the 2-cm margin cm measured around primary tumor for prevent recurrence, because of persistent tumor cells

(Kamstock et al., 2011). Recently, the Tyrosine Kinase inhibitor (TKI) has been used for MCT, nevertheless, the successful treatment appears in only *c-KIT* mutation MCTs (Webster et al., 2006).

Nowadays, in many cancer (such as human prostate gland cancer), the recurrence and metastasis after treatment have been explained by the hypothesis of the CSCs existence (Kreso and Dick, 2014). The Oct-4 transcription factor is one of the major embryonic transcription factors that control self-renewal and pluripotency in embryonic stem cell and many CSC cancers (Beltran et al., 2011). For MCTs, there are evidence of Oct-4 transcription factor expression (Webster et al., 2007b). There is a high possibility that MCTs possess the pluripotency and stemness in term of putative CSCs in the MCT cells population.

5. The characteristic and property of CSCs

The property of CSCs included Self-renewal, pluripotency, High tumor propagation and chemotherapy and radiation resistance.

Self-renewal

Self-renewal is an ability to give rise many another cells. The normal stem cells have ability of self-renewal that can generate new population by have balance between symmetry and asymmetry division. In symmetry division, one stem cell can produce two daughter stem cells or two progenitor cells (cells that develop to differentiated cells). Whereas asymmetry division, each stem cell can generates one daughter stem cell and one progenitor cells. Symmetry division can be seen in early stage of embryonic division while asymmetry division appears at the nearly last stage of embryo together with the ability of proliferation was gradually reduced (Edwards et al., 2010). On the other hand, they switch between asymmetric and

symmetric modes of division for control homeostasis (Orkin and Zon, 2008). Example, the switch of asymmetric cell division in neural stem cells of *Drosophila* to tumor-initiating cells (Castellanos et al., 2008).

Pluripotency

Pluripotency means the ability of progenitor cells to generate various cell types. The progenitor cells of cancer stem cells can generate various types of cancer (Kreso and Dick, 2014). For examples, putative CSCs in chronic myeloid leukemia have a normal hematopoietic stem cell (HSC) that can altering the HSC to myeloid lineages (Dazzi et al., 2000) or pancreatic CSCs can produced subpopulations into immunodeficient mice (Raj et al., 2015).

High tumor propagation

CSCs can migrate to the new sites by propagation or metastasis (Dalerba et al., 2007; Merlos-Suarez et al., 2011; Baccelli et al., 2013). The alternative of CSCs genetic and/or epigenetic mechanisms lead to metastatic. For examples, dysfunction of periostin (POSTN) a component of the extracellular matrix) and Wnt ligand has been increased the fibroblasts formation that led to the expansion of cancer cells. At the first time, pancreatic cancer was used as a model for CSCs metastasis. It is concluded that the primary pancreatic cancer migrated to the other areas (Hermann et al., 2007).

Chemotherapy and radiation resistance

CSCs composed of many structure and function that protect them from antigens. For example, Reactive Oxygen Species (ROS) is a cell membrane enzyme that is produced by normal metabolism of oxygen, over expression after received chemotherapy and radiation treatment leading DNA repair, reduced apoptosis and increase of angiogenesis (Ischenko et al., 2009; van

Staveren et al., 2009). In many studies, many markers increased when received chemo or radio – therapy such as the expression of *CD133+* expression in human glioma cell after radiation (Koch et al., 2013), over expression of *CD271* (Li et al., 2015) and *Oct-4* and *Nanog* expression in embryonic carcinoma stem cell (Rad et al., 2015).

6. Transcription factor

Transcription factors regulate embryonic stem cell self-renewal and differentiated. Basic transcription factors include *Oct 4*, *Nanog*, *Klf*, *Sox-2* and *c-Myc* (Torres-Padilla and Chambers, 2014). The *Oct 4*, *Nanog* and *Sox-2* are three major transcription factors that are expressed in both CSCs and embryonic stem cells (ESCs) that play important roles to maintain the pluripotence and self-renewal. In normal stem cells, *Nanog*, *Sox-2* and *Oct-4* work co -operating by *Oct-4* and *Sox-2* bind together at the promoter region of *Nanog* in living mouse and human ESCs (Torres-Padilla and Chambers, 2014).

In normal ESCs, *Oct- 4*, *Nanog* and *Sox-2* control the properties of self-renewal and pluripotency and inhibit differentiation. Each of transcription factors regulates differential period at development stage. The *Oct-4* controls pluripotency of inner cell mass at blastocyst stage, low level of *Oct-4* at this stage may be demonstrate of Embryonic stem cells (ES) into thophectoderm. Whereas, *Nanog* high efficiency suppresses embryonic ectoderm differentiation and *Sox-2* represses mesendoderm differentiation (Clarke et al., 2006).

In CSCs, *Oct-4*, *Nanog* and *Sox-2* appears in many human cancers, including oral squamous cell carcinoma, prostate cancer, and breast cancer. The expression of *Oct-4* alone have been reported in various cancer such as murine Lewis lung carcinoma, human oral

squamous cell carcinoma, bladder cancer, and seminoma. Nanog and Sox-2 express in human somatic tumors (Torres-Padilla and Chambers, 2014).

Oct-4

Oct-4 is an octamer-binding transcription factor in the POU (Pit-Oct-Unc) transcription factor family that may be called POU5F1 (POU: domain, class 5, transcription factor). The expression of the *Oct-4* mRNA is common in human and mouse ES cells inner cell mass in blastocyst. *Oct-4* expression prevents stem cell differentiation by co-regulate with other factors, such as *Fgf4*, *Rex1/Zfp42*, and *Sox-2* (Tomioka et al., 2002).

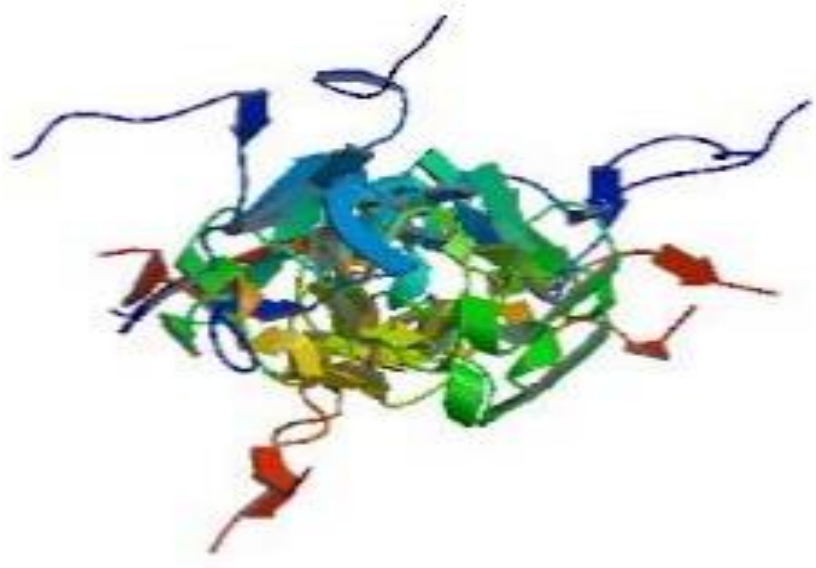


Figure 3. Structure of Oct-4 transcription factor.

(www.phosphosite.org/proteinAction?id=23945&showAllSites=true)

The human *Oct-4* located on chromosome 6 and consists of five exon including exon 1 to exon 5. At the end stage of translation, *Oct-4* have been generated to three main variants

including Oct-4A, Oct-4B and Oct-4B1 (Wang and Dai, 2010). Oct-4A and Oct-4B have been shown similarly in exon 2-5 and differences in exon 1 (Liedtke et al., 2008). The self-renewal and pluripotent properties of *Oct4* were regulated in exon 1.

Oct-4A has 360 amino acids protein that found in the nucleus of embryonic and human somatic stem cell. Although it have been not appeared in the non-pluripotent cells, in non-pluripotent cells may be expressed Oct-4A which unknown reason (Wang and Dai, 2010). For Oct-4B, have 265 amino acids and then expressed at low levels in human somatic stem cells, tumor cells and pluripotency adult tissues.

Immunohistochemistry and immunofluorescence techniques can be used to identify Oct-4A and Oct-4B by the differences of the nuclear and cytoplasmic localization. Oct-4A has been appeared in nucleus whereas Oct-4B found in cytoplasm (Liedtke et al., 2008). In addition, Western blot technique can be identified Oct-4A and Oct-4B by detection their product size (Liedtke et al., 2008). However, the *Oct-4* expression at the RNA level may be found the false positive because there are *Oct-4* pseudogenes (Wang and Dai, 2010).

There is an evidence to support that *Oct-4* expression involved tumorigenesis. For examples, the ectopic expression of *Oct-4* in normal breast cells can produced tumor-initiating cells (Beltran et al., 2011). Moreover, *Oct-4* plays an importance role in the survival of a population of CSCs with drug resistance in prostate gland cancers and breast cancers (Linn et al., 2010; Cho et al., 2018). Similarly, in Veterinary Science, many tumors such as MCT, seminoma, and urinary bladder revealed the immunohistochemical expression of Oct-4 (Webster et al., 2007b).

Nanog

Nanog is a homeodomain-containing transcription factor that control self-renewal and pluripotency in inner cell mass (ICM) during embryonic development. It is regulated by the co-operation of *Oct-4* and *Sox-2* (Cavaleri and Scholer, 2003). It contained 305 amino acids and can be divided into three regions; N-terminal (amino acid 1–95), homeobox domain (amino acid 96–155), and C-terminal (amino acid 156–305) regions (Gehring et al., 1994). *Nanog* has highly diversified among vertebrates and shares high similarity among species.

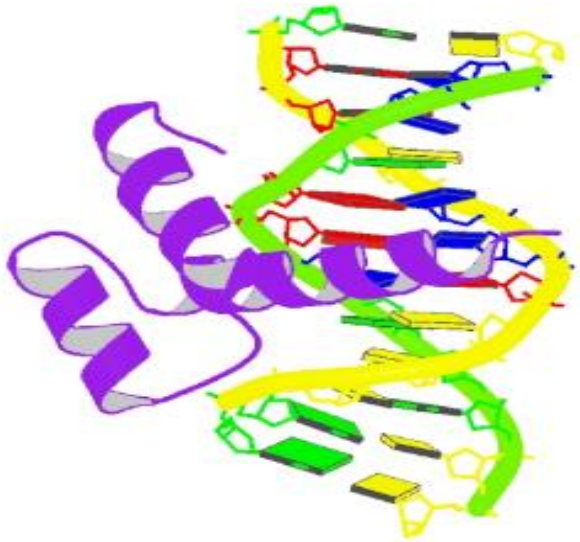


Figure 4. Structure of Nanog transcription factor.

(https://www.rcsb.org/structure/4rbo)

In tumorigenesis, the *Nanog* expression involved cell proliferation (Jeter et al., 2015). Many studies found that over expression of Nanog can be induced tumor-initiating cells while many studies suggested that *Nanog* alone can not induced tumor formation (Lu et al., 2014).

Recently, the expression of *Nanog* has been shown in many cancer including liver tumor , colorectal cancer (Lee et al., 2011; Ibrahim et al., 2012), renal carcinoma , canine mammary tumor (Zamani-Ahmadmahmudi, 2016) and canine cutaneous mast cell tumor (Joselevitch et al.,

2018). The *Nanog* expression were used in clinical for diagnosis, prognosis and therapy. For examples, there have been suggested the *Nanog* marker for detected squamous cell carcinoma, non –small cell lung cancer, prognostic lung cancer, glioblastoma, breast cancer and Ewing's sarcoma (Du et al., 2013; Jeter et al., 2015).

However, the expression of *Nanog* in cancer stem cells have been presented about 2% of the total cancer cell population (Ibrahim et al., 2012).

Sox-2

Sox2 is a DNA-binding protein of the high-mobility group transcription factors (HMG family). *Sox2* is expressed during blastocyst stage and play role at later in development and in adult stem cells. In normal ESCs, *Sox-2* co-operated with *Oct-4*. Depression *of Sox-2* may lead to lethal of mouse embryos, fail to fully develop or become to differentiate (Avilion et al., 2003) .

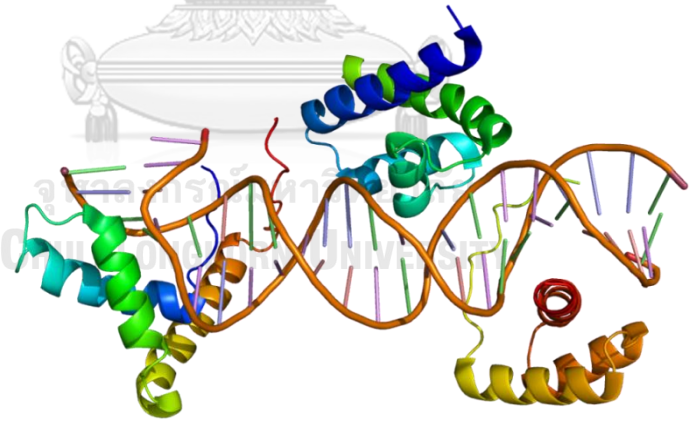


Figure 5. Structure of Sox-2 transcription factor.

(https://upload.wikimedia.org/wikipedia/commons/0/0f/Protein_SOX2_PDB_1gt0.png)

Sox-2 is a one major of pluripotency-associated factors that involved self-renewal and maintenance stemness of embryonic stem cell, pluripotent stem cells formation (iPSCs) and tumor development (Mamun et al., 2018).

In previous studies, the *Sox-2* was overexpressed in many cancers. For examples, the expression of *Sox-2* associated with metastasis stage of pancreatic and prostate cancers (Sattler et al., 2000; Sanada et al., 2006). Additionally, *Sox-2* overexpressed in human small cell lung cancer (Gure et al., 2000), basal cell like breast carcinomas (Rodriguez-Pinilla et al., 2007), and canine mammary tumor (Zamani-Ahmadmahmudi, 2016).

c-Myc

The c-Myc is an oncoprotein that encoded transcription factor. The *c-Myc* control self renewal and pluripotency of stemness cell and play role in proliferation and growth of normal and neoplastic cells such as canine prostatic cancer (Fonseca-Alves et al., 2018). Mutation of *c-Myc* cause of tumors formation and correlated with advanced malignancy and poor prognosis (Vita and Henriksson, 2006). However, there are reports that the inhibition of *c-Myc* expression in mouse bone marrow does not prevent proliferation of haematopoietic stem cells (HSCs) (Wilson et al., 2004).

KLF-4

Kruppel-like factor 4 (KLF4) is the zinc finger transcription factor in Kruppel family. The encoded protein play role in proliferation and differentiation in embryonic stem cells (ECSs) which used for indicator of stemness (Black et al., 2001; Kaczynski et al., 2003). In addition, it can generates apoptosis in case of DNA damage by control the G1-to-S transition cell cycle. Moreover, Klf-4 involved tumor formations. For example, in veterinary science, it have been suggested that Klf-4 expression presents in many tumors such as canine mammary tumor (CMT)

(Yu et al., 2013). Recently, many studies have recommended that downregulation of Klf-4 expression reduced CMT cell proliferation (Tien et al., 2015).

7. Methods for the detection of transcription factors genes and proteins

Aim of this study is the detection of putative CSCs in MCT tissue. The expression of transcription factors Oct-4, Nanog and Sox-2 genes and proteins have been determined by many methods;

7.1 Polymerase chain reaction (PCR)

PCR is a technique in molecular biology that amplifying particular segments of DNA *in vivo* (a thermal cycler) by imitated the replication in natural organisms. One DNA molecule can amplified to million copies by the enzyme DNA polymerase. The enzyme DNA polymerase are the protein that produce from organism which used for synthesis DNA from deoxynucleotide substrates. The DNA polymerase will be added nucleotides to the 3` end of primer sequence at annealed stage (Rahman et al., 2013).

RT-PCR (Reverse Transcription PCR) is the method that detect mRNA expression by convert mRNA to cDNA by Reverse Transcriptase and Quantitative real -time PCR (Real Time , PCR, qRT-PCR or RTq-PCR) is the method that amplify RNA or cDNA within the thermocycler with using an optical system to capture fluorescence that bind to the segment of PCR products. This method can be measured the amount of amplified product in real time during each cycle (Rahman et al., 2013).

However, the PCR technique have some limitation such as necessary in order to generate the primer, DNA polomerase may be cause of mutation in the PCR fragment, and easily to the contamination of DNA (Rahman et al., 2013).

7.2 Immunohistochemistry technique

Immunohistochemistry (IHC) is the biological technique on detecting the antigen in cells and

tissues. This technique is developed from the antigen-antibody binding reaction. The antigens or cellular components can bind with the specific antibody. The antibody-antigen binding complex can be visualized by enzymes, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) to catalyze a color-producing reaction. IHC requires the basic components including, primary antibody binding to specific antigen, secondary enzyme-conjugated antibody. And the chromogen, which is the enzyme catalyzing to generate the color (Matos et al., 2010)

7.3 Intranuclear Immunocytofluorescence (IF)

Intranuclear Immunocytofluorescence is the method that evaluated the expression levels of proteins. IF was used for tissue sections, cultured cells or individual cells immunostaning that form of immunohistochemistry based on the use of fluorophores to visualize the location of antigen-antibody complex. The intranuclear staining involves the process of fixation and permeability before stained with fluorescent-labeled antibodies. This method requires specific antibodies. But the antibodies bound with the high concentration of antigens, it cannot penetrated into the nucleoli (Svistunova et al., 2012).

7.4 Western blot technique

Western blot is a gold standard method that use for separation and identification of proteins in qualitative and quantitative level. The total protein is separated through gel electrophoresis. Each protein is subsequently separated to each band base on its molecular weight. These proteins will be then transferred to a membrane. The interesting protein is identified with the specific antibodies. The positive control protein should be demonstrated in visible band (Mahmood and Yang, 2012). There are three detection methods; which are colorimetric (Enzymatic), chemiluminescence and Fluorescence. The disadvantage of colorimetric method is low sensitivity and require high concentration of protein (Bass et al., 2017).

7.5 Surface Plasmon Resonance technique (SPR)

The Surface Plasmon Resonance (SPR) is a technique that detects the refractive index changed on the surface of thin layer (such as gold, silver and aluminium films. When the layer responses to the biomolecule and reaction on the SPR surface. The nanoparticles of gold film will be stimulated by the incident light, light absorption, refraction and reflection. However, there is an optimal angle that leading surface plasmon resonance (oscillation of electron), so called the Surface Plasmon Resonance angle (SPR angle). SPR angle is the optimal angle that leading minimal light refraction that would be detected by machine. The SPR technique can be determined molecular interaction such as antigen-antibody complex and protein – protein interaction. When, the macromolecule flow to the surface SPR, result of the exchange of SPR angle that can be detected by monitoring the reflected light intensity (Nguyen et al., 2015) . However, the limitation of SPR are the high sensitivity that leaded to the false positive (Nguyen et al., 2015) .

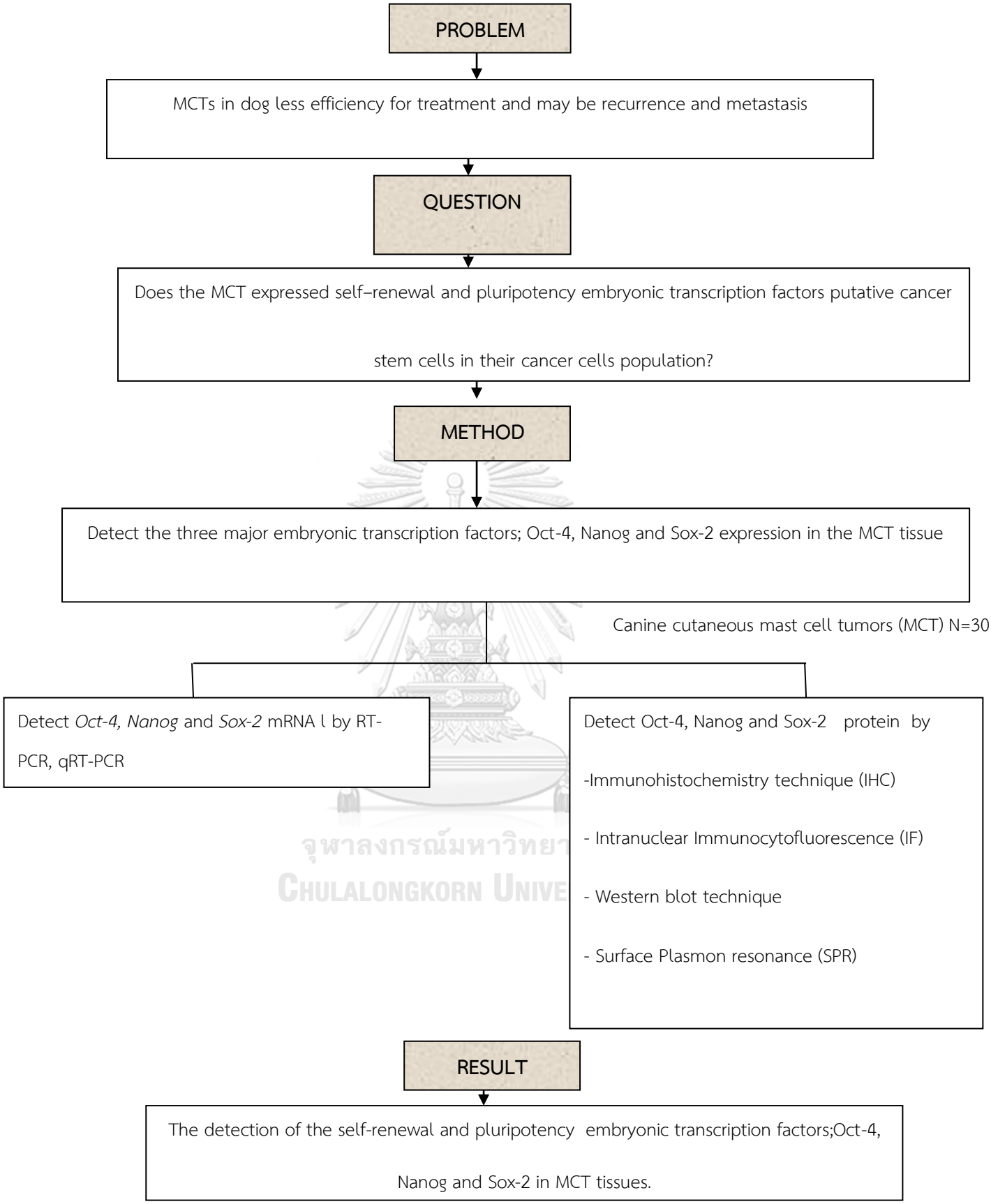


Figure 6. Conceptual framework and research outline of this study

CHAPTER III

MATERIALS AND METHODS

1. Samples collection and experimental design:

MCT affected dogs were obtained from Oncology Unit, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. The MCT suspected dogs were subsequently screened and confirmed by fine-needle aspiration (FNA) followed by Giemsa staining. Thirty MCT tissue samples were collected from the MCT affected dogs in operating room. The consent forms were signed by the dog owners and the research protocol (No.1631055) was approved by the animal ethic committee, Faculty of Veterinary Science, Chulalongkorn University following the Guide for the Care and Use of Experimental Animals, National Research Council of Thailand (NRCT, 2017). All clinical and physical examinations data of MCT dogs were recorded including age, gender, breed, size, location of tumors, clinical history and duration of recurrence after treatment. Blood samples were collected and therapeutic protocols were recorded. The MCT tissues were divided into three parts; part one were then fixed in 10% neutral buffered formalin (NBF) through routine histopathology process for grading by Patnaik's and Two-tier grading system (Patnaik et al., 1984; Kiupel et al., 2011). All tissue samples in paraffin blocks were detected Oct-4 Nanog and Sox-2 protein expression by IHC. Part two, the fresh samples weresubmitted for RT-PCR and qRT-PCR examination. The tissues were stored in RNA later solution and kept at 4°C for 2 hrs followed by -20 °C for 2 hrs. After that the samples were kept in -80 °C until used. The other part, MCT cells were isolated from fresh tissues for Intranuclear Immunofluorescence, Western blot and SPR techniques.

2. RT-PCR and qRT-PCR studies:

2.1 RNA extraction and purification :

One gram of MCT tissue was minced into a small pieces then total RNAs were subsequently extracted using RNA mini kit according to the manufacture's protocol (PureLink RNA Mini Kit, USA). In brief, all cells were lysed in lysis buffer then RNA supernatant was transferred to the Spin Cartridge. Total RNAs was trapped, washed and eluted in a small concentrated volume. The total RNAs were treated with DNase in order to remove contaminated DNA. Purified RNAs were stored at -80°C.

2.2 cDNA synthesis and PCR amplification:

First stranded cDNA was converted from 1 μg total RNAs using cDNA Revertra Ace® qPCR RT Mastermix (Toyobo®, Japan). The RNA was incubated at 65°C for 5 min. Then, the cDNA was synthesized by adding 2 μL of co-reaction 5x RT Master Mix and adjusted nuclease free water to be a final volume of 10 μL. The mixture was incubated at 37°C and 98 °C for 60 min and 5 min, respectively. The PCR was done by following a protocol of KOD FX Neo kit (Toyobo®, Japan). The cDNA was mixed with 2 x PCR Buffer for KOD FX Neo, 2 mM dNTPs, forward primer, reverse primer and DNA Polymerase. The PCR products were amplified by PCR machine (GS1, G-STORM). The primer sequences were shown in Table 1.The PCR running cycles were consisted of: 1 cycle at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 sec., annealing at (55°C for *c-Kit* and β-actin, 60°C for *Oct-4*, 50.3°C for *Nanog* and, 58°C for *Sox-2*) for 30 sec. and extension at 72°C for 30 sec. The PCR products were separated in 2% agarose gel electrophoresis. The PCR products were purified by a QIAquick PCR purification kit (Qiagen®, USA) and then were submitted for sequencing. Canine keratinocyte cell line (line (CELLnTEC

Advanced Cell Systems, Switzerland) and deionizing water were used for positive and negative control, respectively.

This experiment, a *c-Kit* mutation was determined from genomic DNA sample using a pair of *c-kit* primer (table 1). Genomic DNA was extracted and purified from each MCT samples by the commercial DNA isolation kit (Ultraclean Tissue & Cells DNA isolation kit, Mobio, USA). The PCR's protocol was performed followed the School of Veterinary Medicine, Michigan State University with some modification

(Sailasuta et al., 2014). The DNA of normal mast cell was a 191 bp while mutated DNA was 250 bp and/or 300 bp.

2.3 qRT-PCR amplification :

Gene expression was quantitated by using KAPA SYBR®FAST qPCR Master Mix (KAPA BIOSYSTEM®, USA). Five ng of cDNA were mixed with 2 x KAPA SYBR®FAST qPCR Master Mix, forward, reverse primer. The primer sequences were shown in Table 1. Quantitative real time PCR was performed using ESCO® spectrum 48. Each reaction was performed in 3 independent runs. Relative quantitation was determined by delta threshold cycle (ΔCt).

Table 1 primer used in this study

Genes	Primer sequence	Amplicon size (bp)	Reference
Oct-4	*F*: 5'-GAGTGAGAGGCAACCTGGAG-3' *R*: 5'-GTGAAGTGAGGGCTCCC ATA-3'	437	(Guercio et al., 2012)
Nanog	F: 5'-GGACCCTTCTCCAATGCC -3' R:5'-ACCGTCCTCATCTTCTGTTT-3'	348	Primers premier Version 6.0, USA
Sox-2	*F*: 5'-AGTCTCAAGCGACGAAAAA-3' *R*: 5'-GCA GA A G C CTCCTCTTGAA-3'	142	(Guercio et al., 2012)
c-Kit	*F: 5'-* CAA ATC CAT CCC CAC ACC CTG TTCA-*3'* *R: 5'*-CAC TTT CCC GAA GGC ACC AGC ACCCA-*3*	191	(Sailasuta et al., 2014)
β- actin	*F*: 5'-TGTTGCCCTAGACTTAGACTTCGAGCA-3' *R*: 5'-GGACCCAGGAAGGAAGGCT-3'	145	(Endo et al., 2013)

3. Immunohistochemitry study:

All tissue samples in paraffin blocks were cut into 5 μm in thickness and place on the coated- silane slide. The tissues were fixed by heated in an oven at 60℃ for 45 min and then deparaffined before incubated with citrate buffer pH 6.4 antigen retrieval solution (Dako Cytomation, Carpinteria, CA) in microwave at median high temperature for 10 min. Subsequently, tissue slides were washed with 1x phosphate buffered saline (PBS) and blocked endogenous peroxidase with 3% hydrogen peroxide in methanol for 5 min. Non specific binding sites were

blocked by 1 % Bovine serum albumin (BSA) follow by incubated with primary antibodies: Mouse anti-human Oct-4 monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology, INC. (Oct-3/4 (C-10): SC-5279), Mouse anti- human Nanog monoclonal antibody (1:100 dilution; RayBiotech: 119-13641) and Mouse anti- human Sox-2 monoclonal antibody (1:100 dilution; BD bioscience: 560291) at 4 ℃ overnight. Following, the secondary antibody conjugated (Envision^{TM}) were added and incubated in for 45 min. After wash with 1x PBS three times and then chromogen were added. The reaction were visualized with a 3,3′-diaminobenzidine tetrahydrochloride substrate (DAB) and then stopped reaction in distilled water. The slide were counterstained with Mayer's hematoxylin, dehydrated and mounted. The canine testis tissues were used for positive controls for Oct-4, Nanog and Sox-2. For immunopositivity, Oct4, Nanog, and Sox-2 positivity were appeared in the nuclei, cytoplasm and both nuclei and cytoplasm of the neoplastic cell population. Immunostaining was evaluated by the average percentage of positive mast cell tumors in five high-power fields (HPFs; ×40 objective) (Webster et al., 2007a; Eksiritrirat et al., 2012). The counting areas were high population of MCT cells with less connective tissues and similar in each areas.

4. Intracellular Immunocytofluorescence

The 1 gram of each fresh MCT tissues were immediately minced in to small pieces and then were incubated with 0.05% trypsin-EDTA solution at 37°C for 15 minutes. The samples were stopped reaction by add 1% Fetal Calf Serum (FCS) and centrifuge at 3,000 rpm for 15 min. The isolated MCT cells were determined proportion of viable cells by trypan blue staining. Approximate $1x10^6$ MCT cells were washed with PBS and adjusted volume to 1ml by buffer (BD bioscience®, USA) before increased permeability with permeabilisation buffer (BD bioscience®, USA). After that the samples were incubated in primary antibodies: Mouse anti-human Oct-4

monoclonal antibody conjugated Phycoerythrin (PE), Mouse anti-human Nanog monoclonal antibody conjugated PE, and Mouse anti-human Sox-2 monoclonal antibody conjugated PE (BD bioscience®, USA) for 45 min at 4°C. After that the cells were stained with 4,6 –diamidino-2phenylindole (DAPI). Image were then captured by Fluorescence microscopy (Leiga, German company). The canine keratinocyte cell line (CELLnTECAdvanced Cell System , Switzerland) was used for positive control.

5 . Western blot technique

5.1 protein extraction

Approximate $1x10^7$ of c-KIT positive MCT cells were adde with 1 ml ice cold Lysis Buffer, 1ul protease inhibitor and 5 µl phosphatase inhibitor and 10 µl phenylmethysulfonyl fluoride (PMSF) into 1ml Lysis Buffer, vorticed for 30 sec and repeated the operation for three times. The solution transfer into a new collecting tube, place the tube on ice for 10 min and then vortex for 3-4 times. After that solution were centrifuged at 12,000 rpm for 5min at 4°C, total proteins were evaluated by Bradford assay (Atlasi et al., 2007).

5.2 Bradford assay

The Bovine serum albumin (BSA) was used for standard protein. The BSA was diluted for five dilutions. After that the protein samples were diluted for 100µg/30µl. Then five diluted BSA and each protein samples were added in collection tube for the volume of 30µl. The 30µl H_2O was used for blank tube. After that 1.5 ml of Bradford reagent was added in each tubes and then incubated at room temperature for 5 min. The standard protein, protein samples and blank tube were measured absorbance at 595 nm. The concentration of protein samples were calculated with standard curve of standard protein.

5.3 Protein analysis

The 25 ng of total proteins was separated on polyacrylamide gel (10% SDS-PAGE) and then was transferred to polyvinylidene difluoride nitrocellulose membrane (PVDF membrane; Millipore, Billerica, MA, USA). The non specific protein were blocked with 5% BSA in Tris-buffered saline (TBST). The nitrocellulose membrane were incubated with dilution of primary antibodies: Mouse anti-human Oct-4 monoclonal antibody (1:1,000 dilution; Santa Cruz Biotechnology, INC.Oct-3/4 (C-10): SC-5279), Mouse anti-human Nanog monoclonal antibody (1:500 dilution; RayBiotech: 119-13641) and Mouse anti- human Sox-2 monoclonal antibody (1:1,000 dilution; BD bioscience: 560291) over night at 4°C. After that the nitrocellulose membrane were washed in TBST and then were incubated with secondary horseradish peroxidase-labeled goat polyclonal antibody against mouse (1:4,000 dilution; cat no.: ab97265) for 2 hrs at room temperature. The membrane were incubated with DAB substrate for 1 min at room temperature after wash in TBST. The canine keratinocyte cell line was used for positive control.

6. Surface Plasmon Resonance technique (SPR)

6.1 Modified gold nanoparticles surface by self-assembled preparation (SAM)

After gold film was cleaned with H_2O_2: H_2O and then dried with a flow of N_2.

The gold film was placed to UV ozone for 5 minutes before transfer to mixed SAM (1:1 solution of 11-mercaptoundecanoic acid (MUA) and 3-mercaptopropanol (MPOH) for over night. Before used, the gold film was washed with H_2O and drying with a flow of N_2.

6.2 Step of protein samples analysis

1) Gold nanoparticles were placed on glass and then set wavelength and SPR angle.

2) The primary antibodies: Mouse anti-human Oct-4 monoclonal antibody (1:100 dilution;

Santa Cruz Biotechnology, INC.Oct-3/4 (C-10): SC-5279), Mouse anti-human Nanog monoclonal antibody (1:50 dilution; RayBiotech: 119-13641) and Mouse anti- human Sox-2 monoclonal antibody (1:100 dilution; BD bioscience: 560291) were continuously flowed to the surface of gold nanoparticle and then record the change of SPR angle.

3) Association step: total protein extracted from the MCT samples were

4) flowed to the surface of gold nanoparticle and then recorded the change of SPR angle.

5) Dissociation: PBS was used for flushing through the surface of gold nanoparticle, washing the protein sample and then recorded the change of SPR angle.

6) Regeneration: The primary antibody was washed by using regenerate buffer and then recorded the change of SPR angle.

7) Base line: The exchange of SPR angle after primary antibody was degraded.

(Nguyen et al., 2015).

7. Statistical analysis

The results were analysed by mean and standard deviation. The level of *Oct-4*, *Nanog* and *Sox-2* mRNA and protein expression were performed using one way ANOVA compare the variables between Grade I, II, and III (Patnaik's system) and high and low grade (Two-tier system). Results of immunohistochemistry were evaluated the variables of percentage positive cells between Grade I, II, and III and low and high grade in each antibody by one way ANOVA. All

statistics were calculated by using SPSS version 15 for Windows (IBM Corporation, New York, NY, USA).The level of statistical significance will be set at 0.05 ($p \leq 0.05$) for all tests.

CHAPTER IV

RESULTS

1.MCT case's signalment and histopathology grading

The 30 MCT cases were collected from the MCTs affected dogs, Oncology Clinic, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University (Fig.7). The cases were aspirated for MCT's screening using Giemsa staining. The tumor cells were monomorphous or polymorphous, intracytoplasmic granules or eosinophilic with distinct cytoplasmic border. The nuclei have varied from round to oval (Fig.8A). MCT samples were histopathologically grading by Patnaik's system and Two-tier system. In Patnaik's grading system, Grade I, the cells showed no evidence of mitotic figures (Fig.8B). In grade II, the MCT cells were pleomorphism with mitotic figure 0-2/10 high power fields (HPF) (Fig.8C), and Grade III, MCT cells were contained the large number of mitotic figures (at least 3-6/10 HPF) (Fig.4D) (Patnaik et al., 1984).

For Two-tier grading system (Kiupel et al., 2011), the low grade showed the uniformity neoplastic cells supported by low amounts of collagenous stroma. The mitotic and multinucleated cells were absent (Fig 9A). High grade MCT sample showed the pleomorphic round neoplastic cells with mitotic and multinucleated cells were seen (Fig.9B).

For Patnaik's grading system divided into three grades; grade I (12/30; 40%). Grade II (8/30; 26.67%) and Grade III (10.30; 33.33%). While, Two-tier grading system defined as low grade (20/30, 66.67%); meanwhile, the others were high grade (10/30, 33.33 %) (Table 2).

Thirty MCT dogs were sixteen males (53.33%) and fourteen females (46.67%). The age of these dogs ranged from 7 to 15 years (mean age of 9.43 years). Tumors were located on the

abdomen (n = 8), inguinal (n = 3), thorax (n = 3), forelimb (n=4), hind limb (n=2), elbow (n=1), mandible (1), axilla (2), left hock (1), stifle joint (1), scrotal sac (1), lumbar (1), and tail (1) (Table 2.). The breed of MCT samples included mixed breed (21/30, 70%), Bangkhaw (4/30, 13.33%), Golden retriever (1/30, 3.33%), French bull dog (1/30, 3.33%), Labrador retriever (2/30; 6.67%) and Pug (1/30, 3.33%) (Table 2).

Figure 7. The dogs affected with MCT that were collected for the experiment.
The mast cell tumor was found on shoulder area (left) and scrotal sac (Right).

Table 2 MCT Case description

Case No.	Two-tier grade	Patnaik grading	Sex	Age (year)	Breed	location of tumor
1	L1	Grade I	F	9	French Bulldog	Inguinal
2	L2	Grade I	M	10	Mixed	Mass at left mandible
3	L3	Grade I	M	10	Mixed	Mass at thorax
4	L4	Grade I	F	15	Bangkhaw	Mass at abdomen
5	L5	Grade I	F	10	Mixed	Mass of left forelimb
6	L6	Grade I	F	7	Mixed	Abdominal wall
7	L7	Grade I	M	8	mixed	Right axilla
8	L8	Grade I	M	8	Labrador retriever	Left hind limb
9	L9	Grade I	M	10	Bangkhaw	Mass of ventral abdomen
10	L10	Grade I	M	8	Labrador retriever	axilla
11	L11	Grade I	M	7	mixed	Scrotal sac
12	L12	Grade I	M	11	mixed	Abdominal wall
13	L13	Grade II	F	10	Mixed	Mass of left forelimb
14	L14	Grade II	M	10	Bangkhaw	Mass of ventral abdomen
15	L15	Grade II	F	10	Mixed	Mass of left upper gingival
16	L16	Grade II	M	8	Mixed	Ventral abdomen
17	L17	Grade II	M	12	Mixed	Right inguinal
18	L18	Grade II	F	8	Mixed	Mass of right elbow
19	L19	Grade II	M	9	Mixed	Mass of ventral abdomen
20	L20	Grade II	F	9	mixed	Mass of thorax
21	H1	Grade III	F	10	Mixed	Mass of left inguinal area

Case No.	Two-tier grade	Patnaik grading	Sex	Age (year)	Breed	location of tumor
22	H2	Grade III	F	8	Bangkhaw	Mass of right thorax
23	H3	Grade III	M	10	Mixed	Mass of left hock joint
24	H4	Grade III	F	9	Mixed	Mass at tail
25	H5	Grade III	F	11	Mixed	Mass of left forelimb
26	H6	Grade III	F	7	Mixed	Mass of stifle joint
27	H7	Grade III	M	9	pug	Abdominal wall
28	H8	Grade III	M	10	Golden retriever	Mass of lumbar
29	H9	Grade III	F	10	mixed	Mass of forelimb
30	H10	Grade III	M	10	Mixed	Mass of hind limb

L= low grade, H=High grade

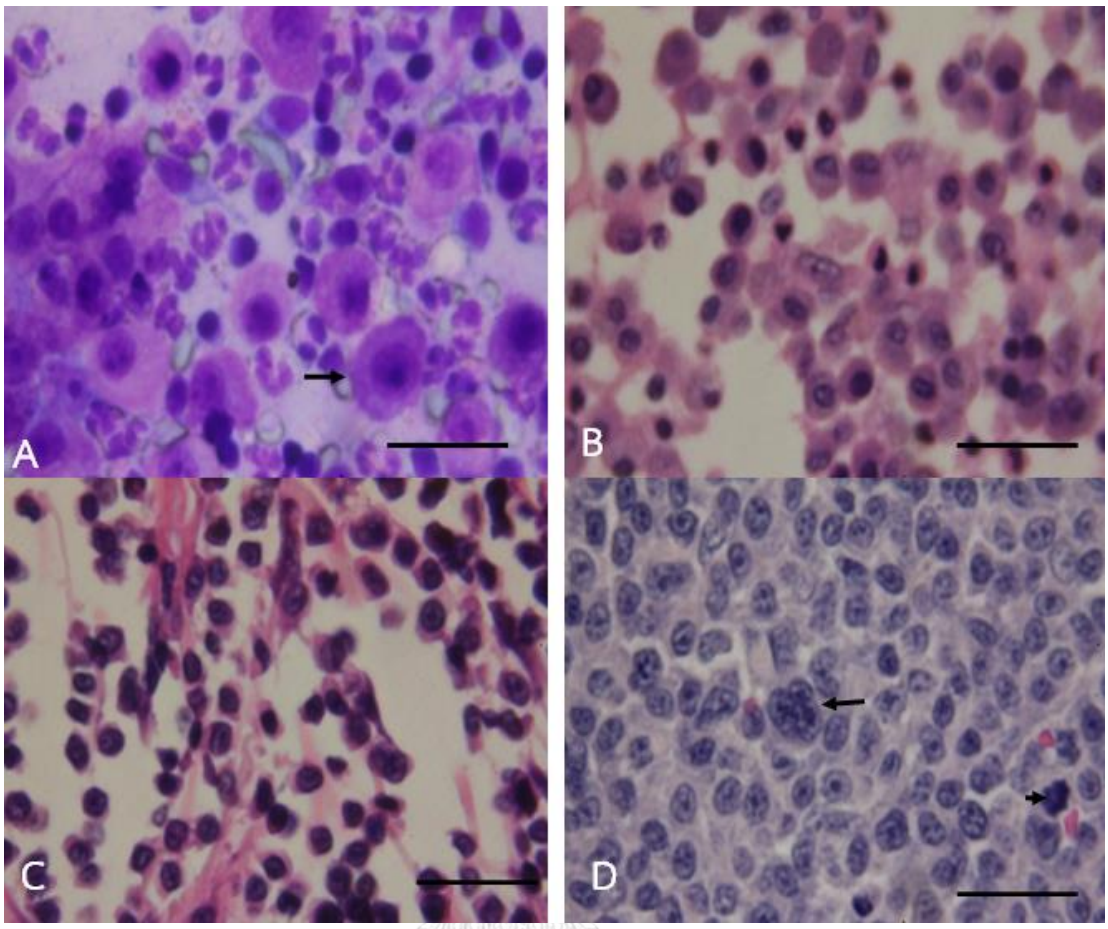


Figure 8. Microscopic findings of the MCT biopsies.

Microscopic findings of the MCT biopsies, Patnaik's grading system A) Fine – needle aspiration of MCT. There are many large round cells with purple staining cytoplasmic granules (arrow); Giemsa staining, Patnaik's grading system; B) grade I has uniformity neoplastic cells and no evidence of mitotic C) grade II, MCTs has pleomorphism with mitotic figure 0-2/10 high power fields D) grade III contain the large number of mitotic at least 3-6/10 HPF) (short arrow) H&E staining Bar= 50μm

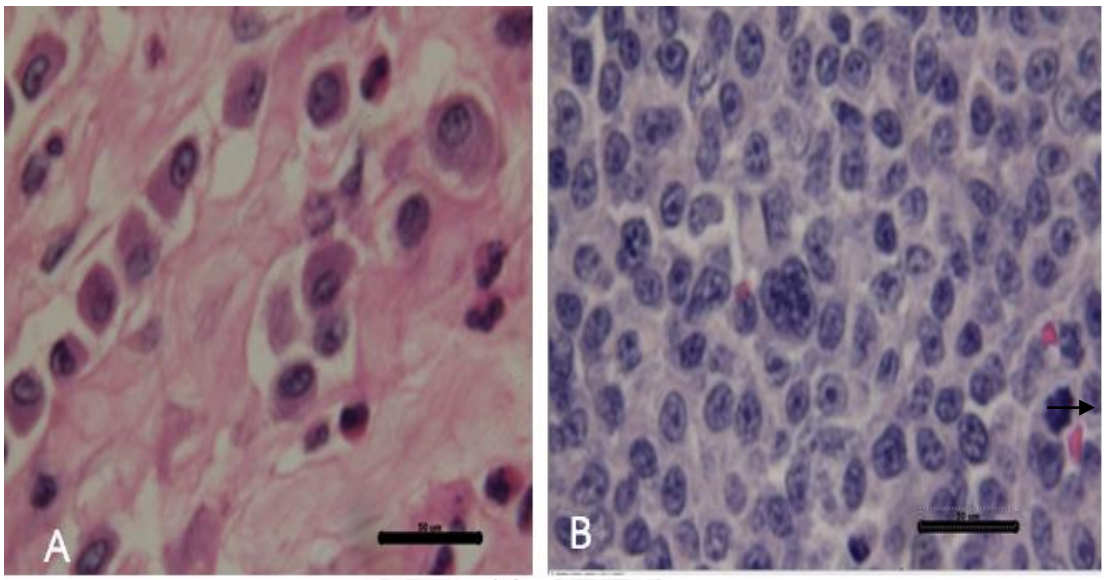
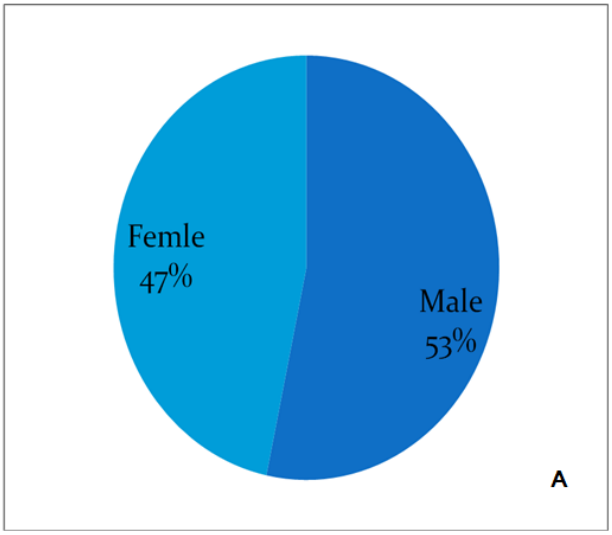
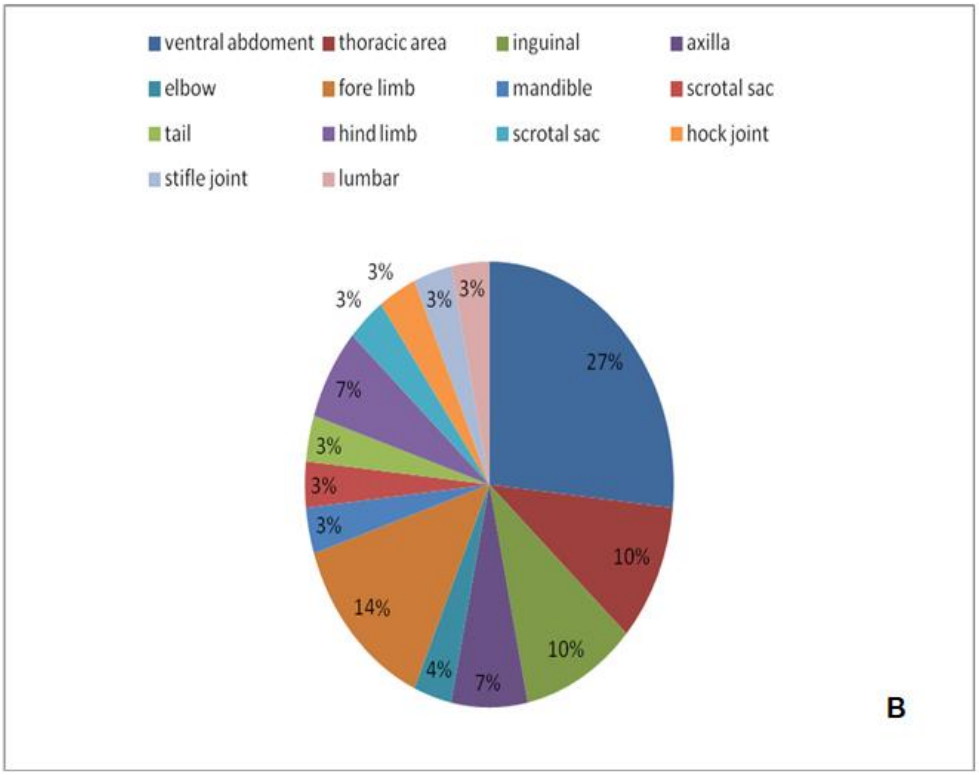


Figure 9. MCT histopathology grading in MCT samples.
Two-tier grading system **A)** Low grade; MCT sample showed the uniformity neoplastic cells supported by low amounts of collagenous stroma. These cells were round to polygonal and contained abundant amphophilic cytoplasm with numerous fine basophilic intracytoplasmic granules, **B)** High grade; MCT sample showed the pleomorphic round neoplastic cells with mitotic and multinucleated cells were seen (arrow). H&E staining Bar= 50 μm


Femle
47%
Male
53%
A


ventral abdoment
thoracic area
inguinal
axilla
elbow
fore limb
mandible
scrotal sac
tail
hind limb
scrotal sac
hock joint
stifle joint
lumbar
27%
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B

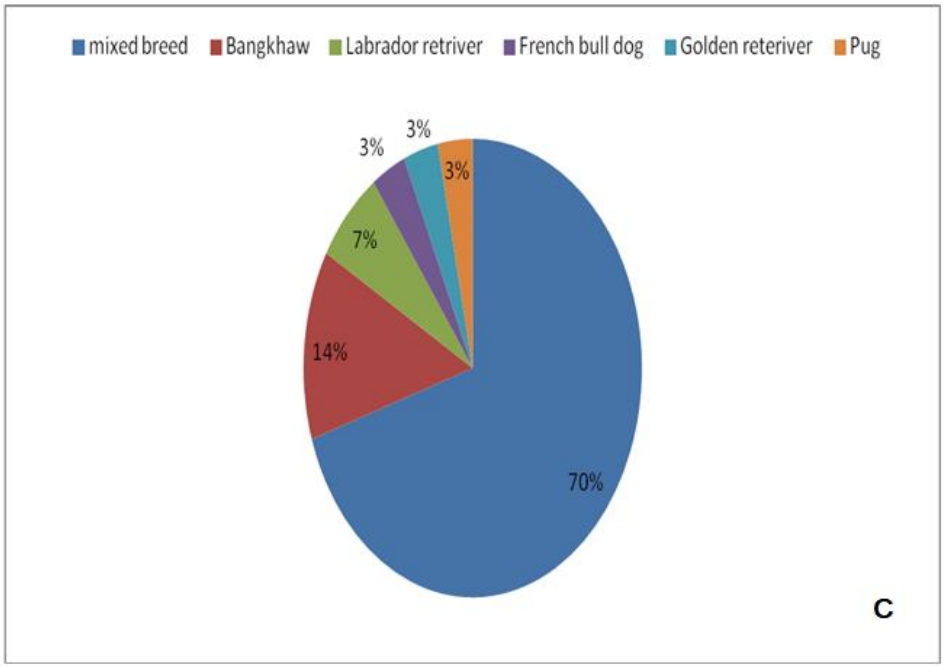


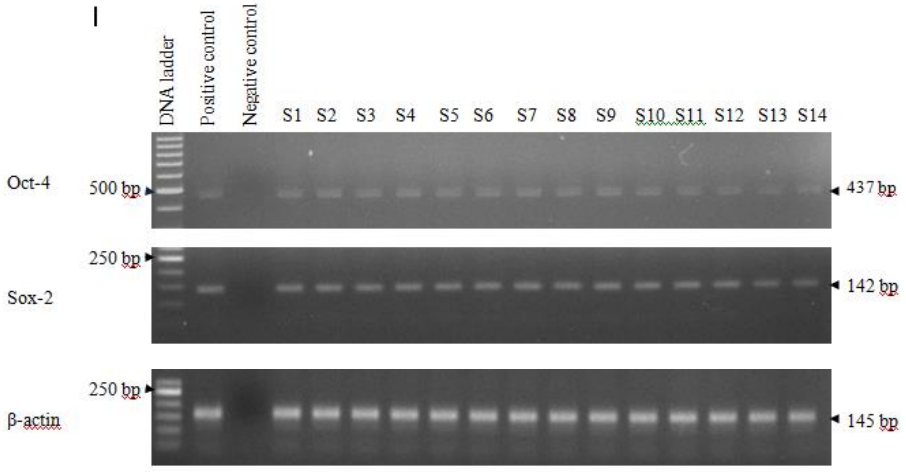
Figure 10. Signalment of MCT samples.
(A) Pie chart of sex ratio that affected with MCT tumors, (B) Pie chart of location of MCT tumors, (C) Pie chart of breed that affected with MCT tumors.

2.The Expression of Transcription factors Oct-4, Nanog, and Sox-2 genes by RT-PCR and qRT-PCR in MCT cases.

All of the 30 MCT samples in this study were evaluated expression of *Oct-4*, *Nanog*, and *Sox-2* at mRNA level by RT-PCR method. The *Oct-4* expression showed in the 2% agarose gel of PCR products, representing at 437 bp (Fig.11). The *Sox-2* expression generated expected PCR product size of 142 bp (Fig.11). While the expression of *Nanog* showed only in positive control (canine keratinocyte cell line) at 348 bp of product size (Fig.12). The same samples in this study were evaluated for *c-Kit* mutation. There were no *c-Kit* or Internal tandem duplication (ITD) mutation in exon11. This results demonstrated the expected PCR product size at 191 bp in all

samples (Fig.12). The PCR products were confirmed sequencing by NCBI blast as shown in Table 3.

The MCT samples were demonstrated the expression of *Oct-4*, *Nanog*, and *Sox-2* by qRTPCR. The results of qRT-PCR showed in Table 4-5, β-actin used for internal control. The delta CT of *Oct-4* was 0.02±6.39, -1.44±2.28, and 2.00±2.49 in grade I, II, and III, respectively. The delta CT of Oct-4 were -0.52±5.17 and 2.00±2.49 in low and high grade, respectively. The delta CT of *sox-2* was 2.95±2.10, 1.97±0.18, and 2.15±2.04 in grade I, II, and III, respectively. The delta CT of *sox-2* was 2.71±1.85 and 2.15±2.04 in low and high grade, respectively. In histopathology grading of MCT, the *Oct-4* gene in high grade tended to be higher than low grade parallelly in grade III also tended to be higher than grade I and II respectively. On the contrary, *Sox-2* expression, in grade III and high grade tended to be lower than grade I and low grade respectively, when compared to internal control. However, the results had no statistically significant for the experiments among the grading system.



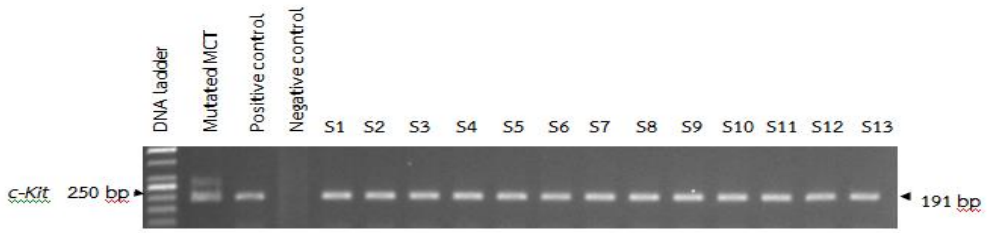


Figure 11. RT-PCR analysis of *Oct-4, Sox-2* and *c-Kit*.

2% agarose gel of PCR amplified *Oct-4* (437 bp), *Sox-2* (142 bp), *c-Kit* (191 bp of normal allele and 300 bp of mutant allele), beta actin (145 bp)

PC= Positive control (keratinocyte cell line), NG=Deionizing water, S1-S9= Low grade MCT samples, S10-S14=High grade MCT samples

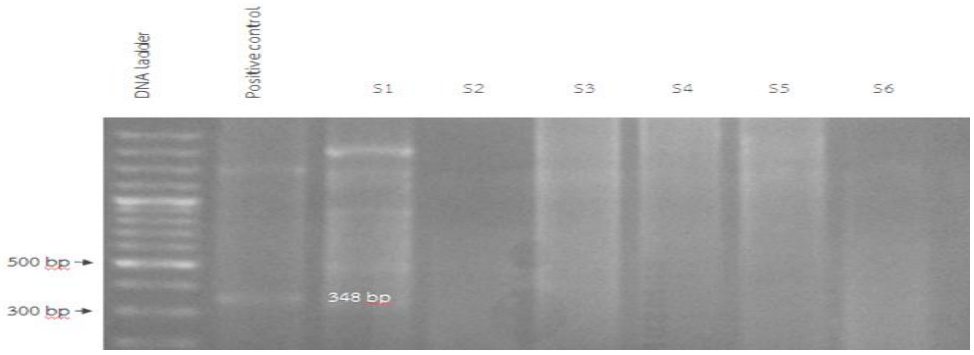


Figure 12. RT-PCR analysis of Nanog

2% agarose gel of PCR amplified *Nanog* (348 bp)

Positive control = keratinocyte cell lineSamples = S1-S6

Table 3. The result of Oct-4, Nanog, Sox-2 and c-Kit sequencing (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

	Description	Max score	Total score	Query Cover	E value	Per. Ident	Accession
Oct-4	PREDICTED *Canis lupus familiaris* POU class 5 homebox 1 (POU5F1) mRNA	222	410	55%	8e-54	95.10%	XM 538830.3
Nanog	PREDICTED *Canis lupus familiaris* nanog homebox (Nanog) mRNA	538	538	92%	5e-149	99.66%	XM 022411387.1
Sox-2	PREDICTED *Canis lupus familiaris* SRP box 2(Sox 2) mRNA	171	171	89	6e-39	95.41%	XM 005639752.3
c-Kit	PREDICTED *Canis lupus familiaris* SRP box 2(Sox 2) mRNA	150	150	59%	1e-32	94.12%	XM 005627970.3

Table 4. Fold change expression of *Oct-4* in MCT samples (Patnaik's system and Two-tier grading system) (30 cases)

Sample	*Oct-4* average Ct	β *Actin* average Ct	ΔOct-4-β *Actin*
Grade I (n=12)	26.82±5.83	26.79±5.18	0.02±6.39
Grade II (n=8)	21.81±5.25	23.26±4.23	-1.44±2.28
Grade III (n=10)	26.00±5.22	24.00±4.39	2.00±2.49
MCT(low grade) (n=20)	24.94±5.99	25.46±5.02	-0.52±5.17
MCT(high grade) (n=10)	26.00±5.22	24.00±4.39	2.00±2.49

Table 5. Fold change expression of *Sox-2* in MCT samples (Patnaik's system and Two-tier grading system) (30 cases)

Sample	*Sox-2 average Ct*	*β Actin average Ct*	ΔCt Sox-2-β Actin
Grade I (n=12)	**23.17±2.97**	**20.07±3.52**	**2.95**±2.10
Grade II (n=8)	**24.49±5.71**	**22.62±5.65**	**1.97**±0.18
Grade III (n=10)	**23.12±2.75**	21.17±2.63	**2.15±2.04**
MCT(low grade) (n=20)	**23.61±3.86**	20.92±4.26	2.71±1.85
MCT(high grade) (n=10)	**23.12±2.75**	21.17±2.63	**2.15±2.04**

Patnaik's grading system

Two-tier grading system

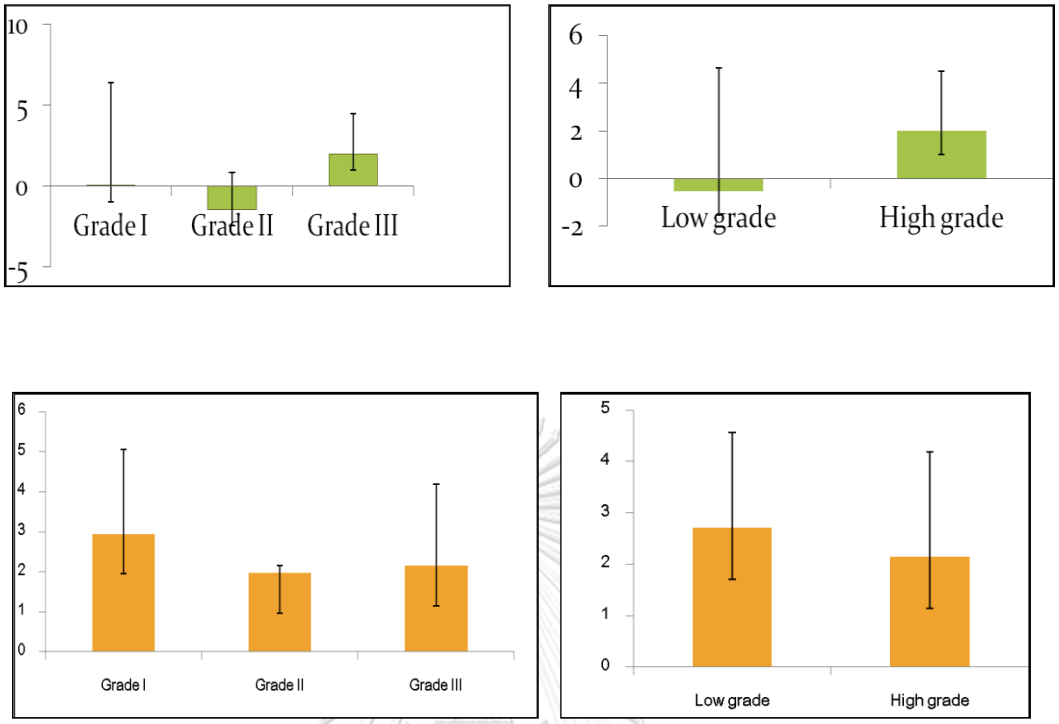


Figure 13. Graph of fold change of *Oct-4* and *Sox-2* in MCT cases.

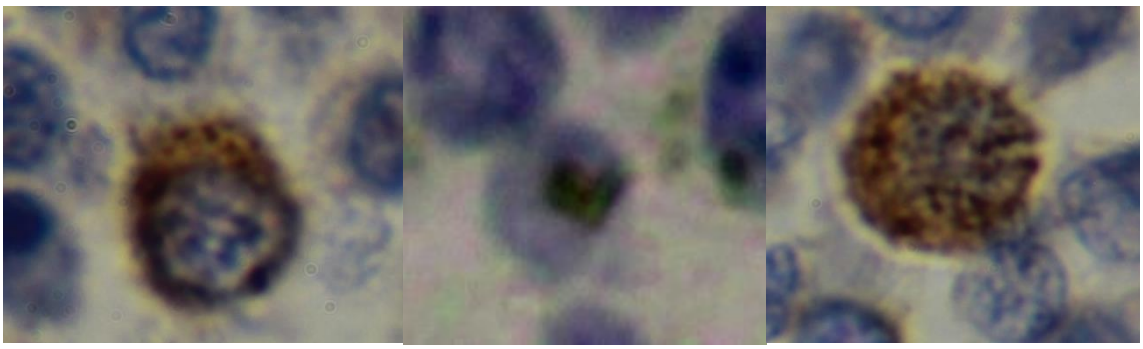
3.The Immunohistochemical Expression of transcription factor Oct-4, Nanog, and Sox-2 proteins in MCT cases

Results of IHC was shown in Table 8. Immunohistochemical staining patterns showed the expression of Oct-4, Nanog, and Sox-2 in the cytoplasmic and nuclear staining of MCT cells (Fig. 14). The staining Intensity of Oct-4, Nanog, and Sox-2 were classified into 4 scores: 0=negative, 1=weak, 2=moderate, and 3=strong. Weak staining intensity (score 1) was defined by pale staining in the nucleus, moderate (score 2) was characterized by stippled or faint intranuclear staining and pale staining in cytoplasm, and strong (score 3) staining intensity was defined as homogenous intense staining of the nucleus and in cytoplasm (Fig. 14). The

positive staining cells in the tumor cell population were then counted in 5 random high-power field (×40) as the criteria followed; each area have similar, has high MCT cell population in selecting area with less connective tissue. The mean percentage of counting cells was given to each case.

For Patnaik's histopathology grading, Oct-4 was positive in 100 % (12/12) in grade I, 87.5 % (7/8) in grade II and 80% (8/10) in grade III. Nanog positive in 91.67 % (11/12) in grade I, 37.5% (3/8) in grade II and 100 % (10/10) in grade III . While, Sox-2 positive in 75 % (9/12) in grade 1, 87.5 % (7/8) in grade 2 and 80 % (8/10) in grade 3 respectively. In Two-tier grading system, the Oct-4 positive-case was 95 % (19/20) in low grade and 80 % (8/10) in high grade. Nanog positive-case was 70% (14/20) and 100-% (10/10) in low and high grade, respectively. While, Sox-2 positive was 80% (16/20) in low grade and 80% in high grades (8/10) (Table 6).

For the number of positive cells per area (56,250μm^2), in Patnaik's histopathology grading, Oct-4 positive cells were 13.5$\pm$6.1 in grade I, 128.47$\pm$75.78 in grade II and 151.53$\pm$31.98 in grade III. Nanog positive cells were 113.06$\pm$21.78 in grade I, 81.2$\pm$85.98 in grade II and 107.47$\pm$28.63 in grade III . While, Sox-2 positive cells were 10.53$\pm$8.56 in grade I, 1.65$\pm$0.46 in grade II and 72.2$\pm$3.11 in grade III respectively. The Two-tier grading system, the Oct-4 positive cells were 82.54$\pm$76.21 in low grade and 151.53$\pm$31.98 in high grade. Nanog positive cells were 109.25$\pm$35.14 in low grade and 107.47$\pm$28.63 in high grade. While, Sox-2 positive cells were 4.34$\pm$5.89 in low grade and 72.2$\pm$3.11 in high grade. The number of Sox-2 positive cells in high grade were significant different higher than in low grade (Fig.14). The positive cells result of Oct-4 and Nanog were not difference in all grades ($p > 0.05$).



Intracytoplasmic staining | Intranuclear staining | Intracytoplasmic and nuclear staining

Figure 14. Immunohistochemical staining patterns.

IHC, DAB, Hematoxylin counterstained, Bar= 50μm

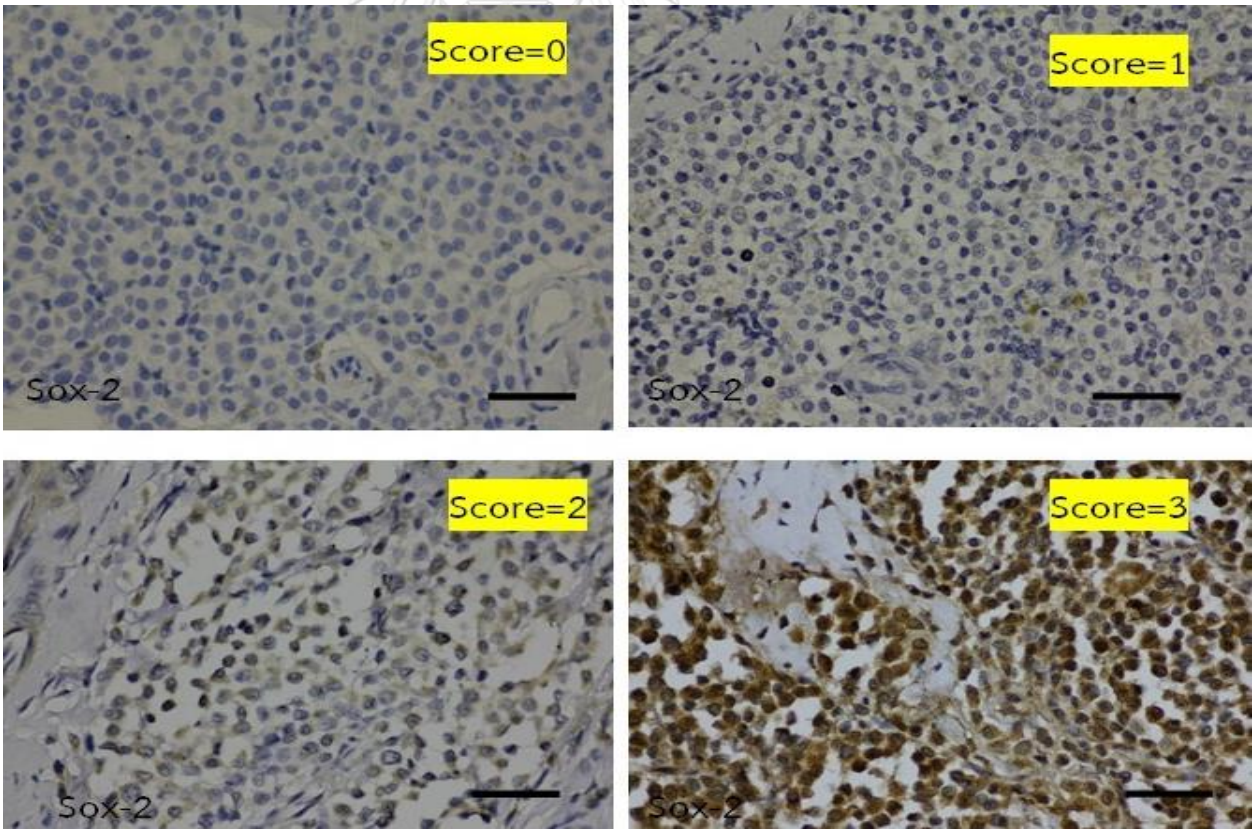


Figure 15. The Intensity of the staining of Oct-4, Nanog, and Sox-2.

The Intensity of the staining of Oct-4, Nanog, and Sox-2 were classified into 4 scores: 0=negative, 1=weak , 2=moderate , and 3=strong. IHC, DAB, Hematoxylin counterstained, Bar = 50μm

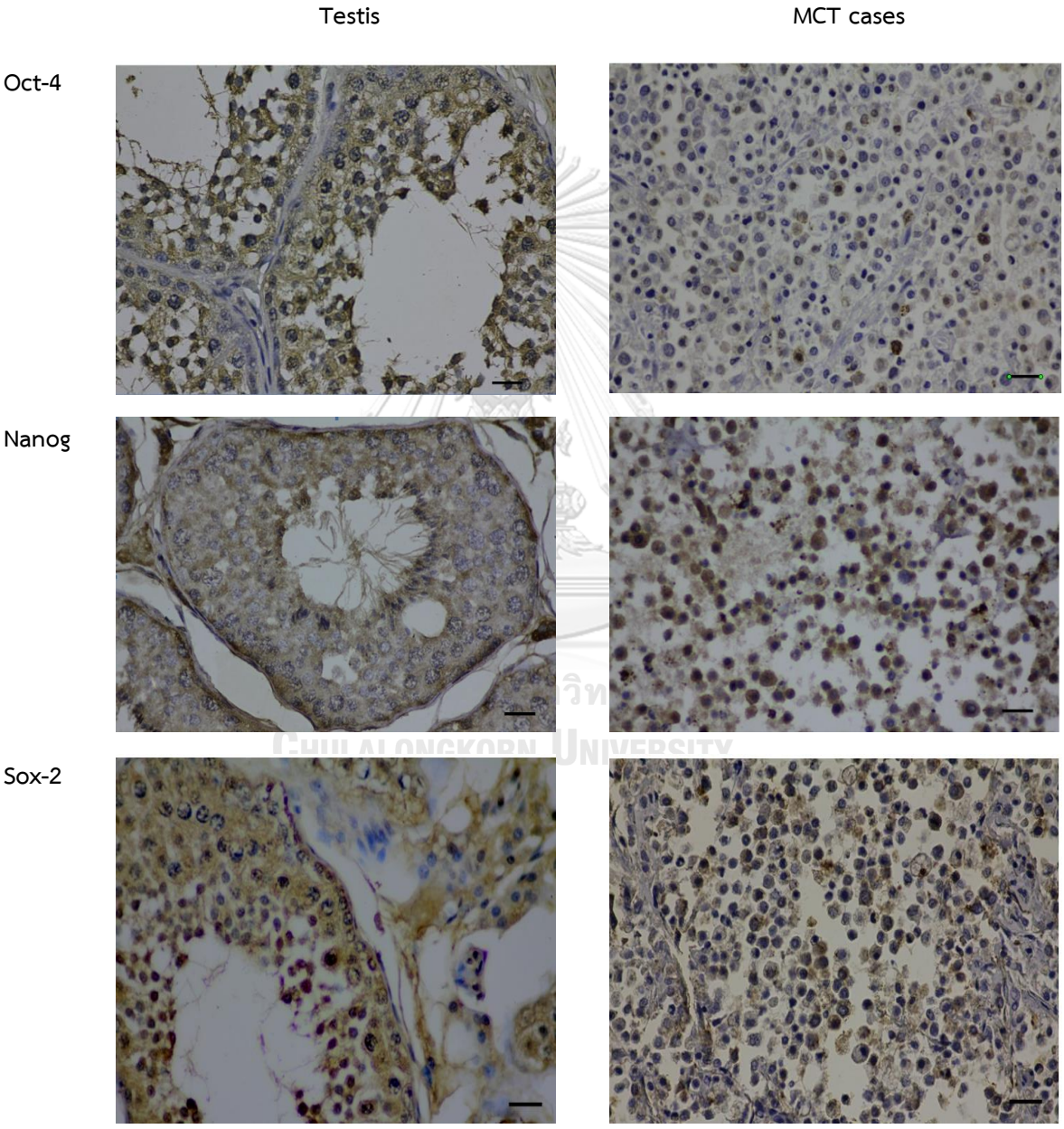


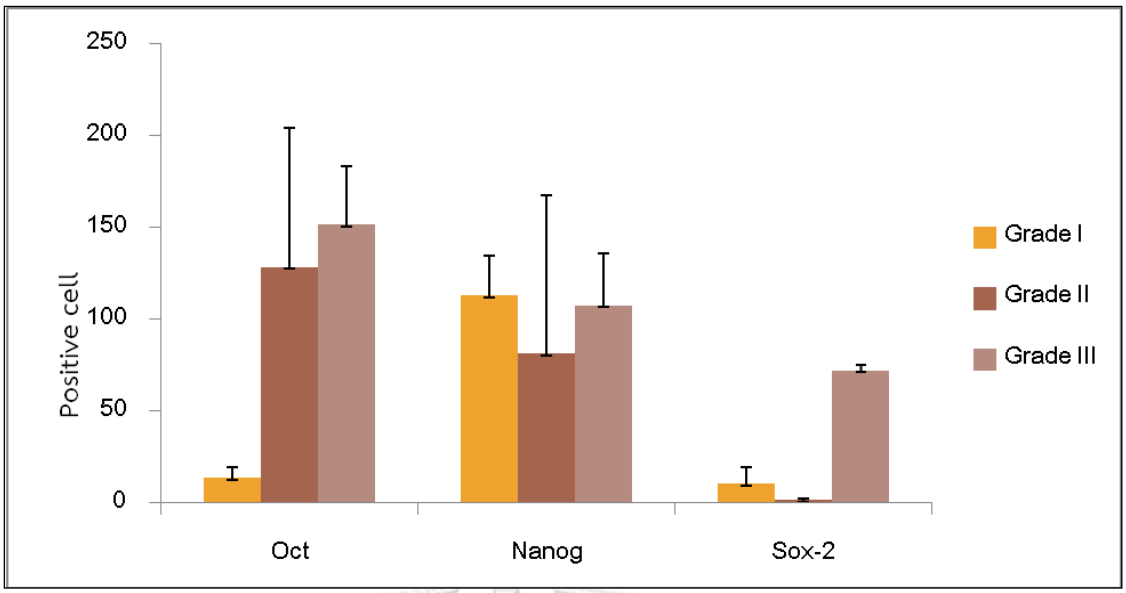
Figure 16. The Immunohistochemical Expression of Oct-4, Nanog, and Sox-2 in MCT samples. Testis was used for positive control for Oct-4, Nanog and Sox-2. Immunohistochemical, DAB, Hematoxylin counterstained, Bar = 50μm

Table 6. The percentage of positive cases in MCT samples.

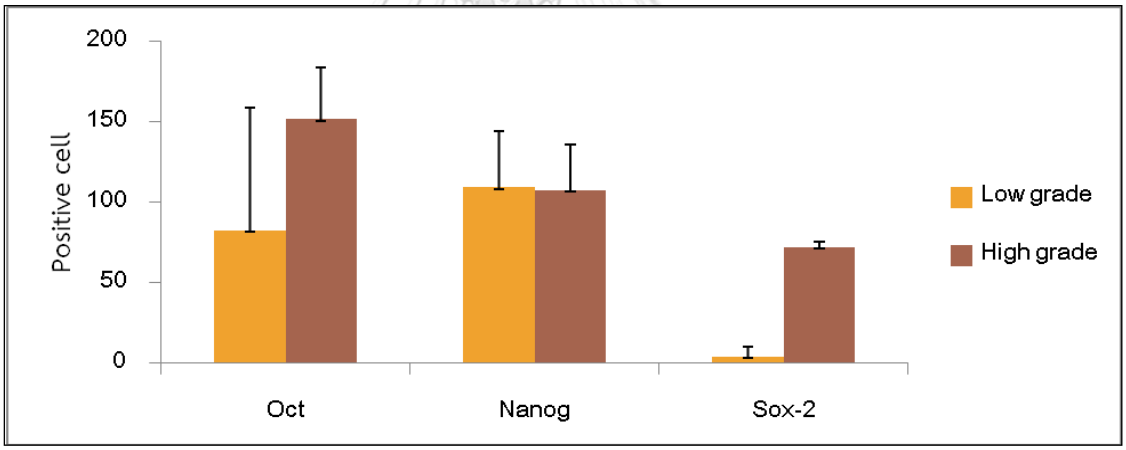
Grading system	**Positive cases (N/Total)(%)**		
	Oct-4	Nanog	Sox-2
Patnaik's system			
Grade I	100% (12/12)	91.67% (11/12)	75% (9/12)
Grade II	87.5% (7/8)	37.5% (3/8)	87.5% (7/8)
Grade III	80% (8/10)	100% (10/10)	80% (8/10)
Two-tier grading system			
Low grade	95% (19/20)	70% (14/20)	80% (16/20)
High grade	80% (8/10)	100% (10/10)	80% (8/10)

Table 7. The number of positive cells per area of Oct-4, Nanog and Sox-2 expression in MCT cases.

Grading system	**Positive cases (N/Total)(%)**		
	Oct-4	Nanog	Sox-2
Patnaik's system			
Grade I	13.5±6.1	113.06±21.78	10.53±8.56
Grade II	128.47±75.78	81.2±85.98	1.65±0.46
Grade III	151.53±31.98	107.47±28.63	72.2±3.11
Two tier grading system			
Low grade	82.54±76.21	109.25±35.14	4.34±5.89
High grade	151.53±31.98	107.47±28.63	72.2±3.11



Patnaik's grading system



Two-tier grading system

Figure 17. Graph of number of positive cells of Oct-4, Nanog and Sox-2 expression in MCT cases.

4.The Demonstration of Intranuclear Immunocytofluorescence (IF) of transcription factor Oct-4, Nanog, and Sox-2 proteins in MCT cases

The MCT samples were demonstrated the Oct-4, Nanog, and Sox-2 proteins by Intranuclear Immunocytofluorescence. The canine keratinocye cell line was used for positive control. The PE-conjugated antibody was used for detected the positive cells, the red color was demonstrated with intranuclear and intracytoplasmic of positive cells. DAPI fluorescent dye was used for nuclear staining in both negative and positive cells which blue color staining .

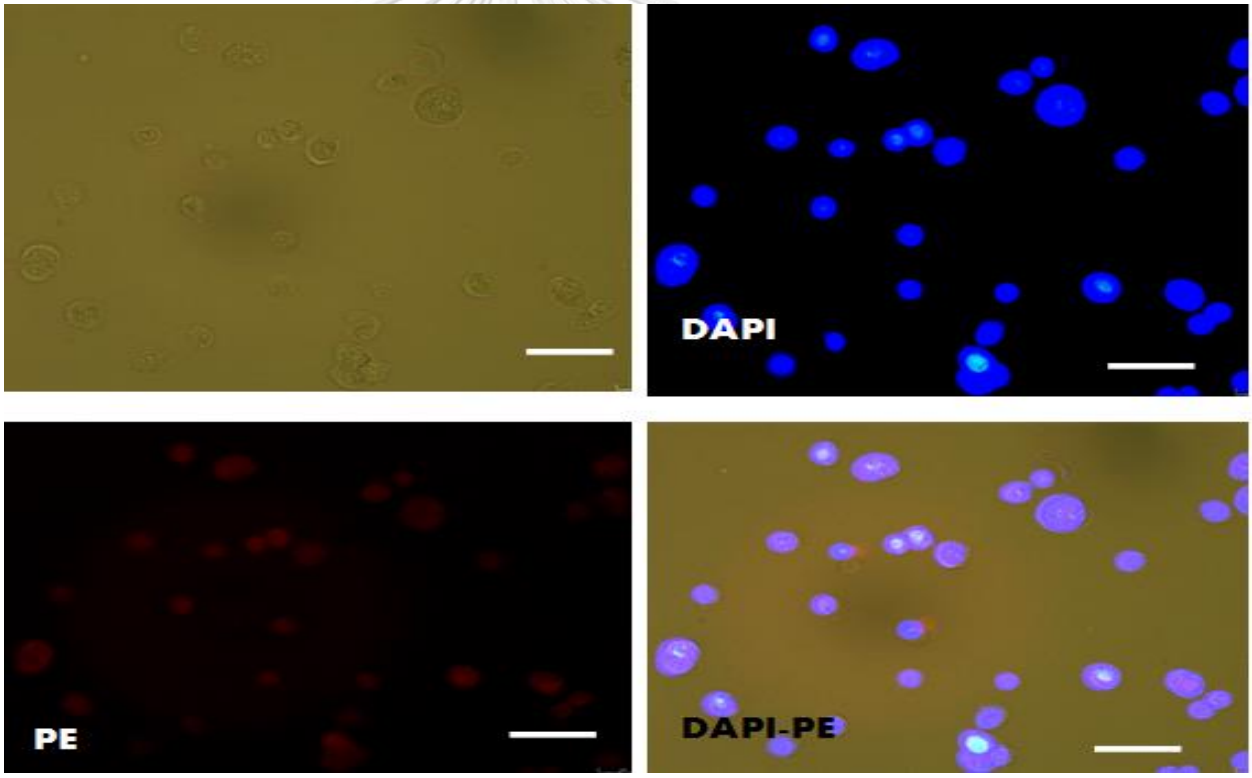


Figure 18. The Oct-4 protein detection in canine keratinocyte cell line.
The Oct-4 protein detection in canine keratinocyte cell line (Positive control). Fluorescence microscope, PE, Bar = 50 μm

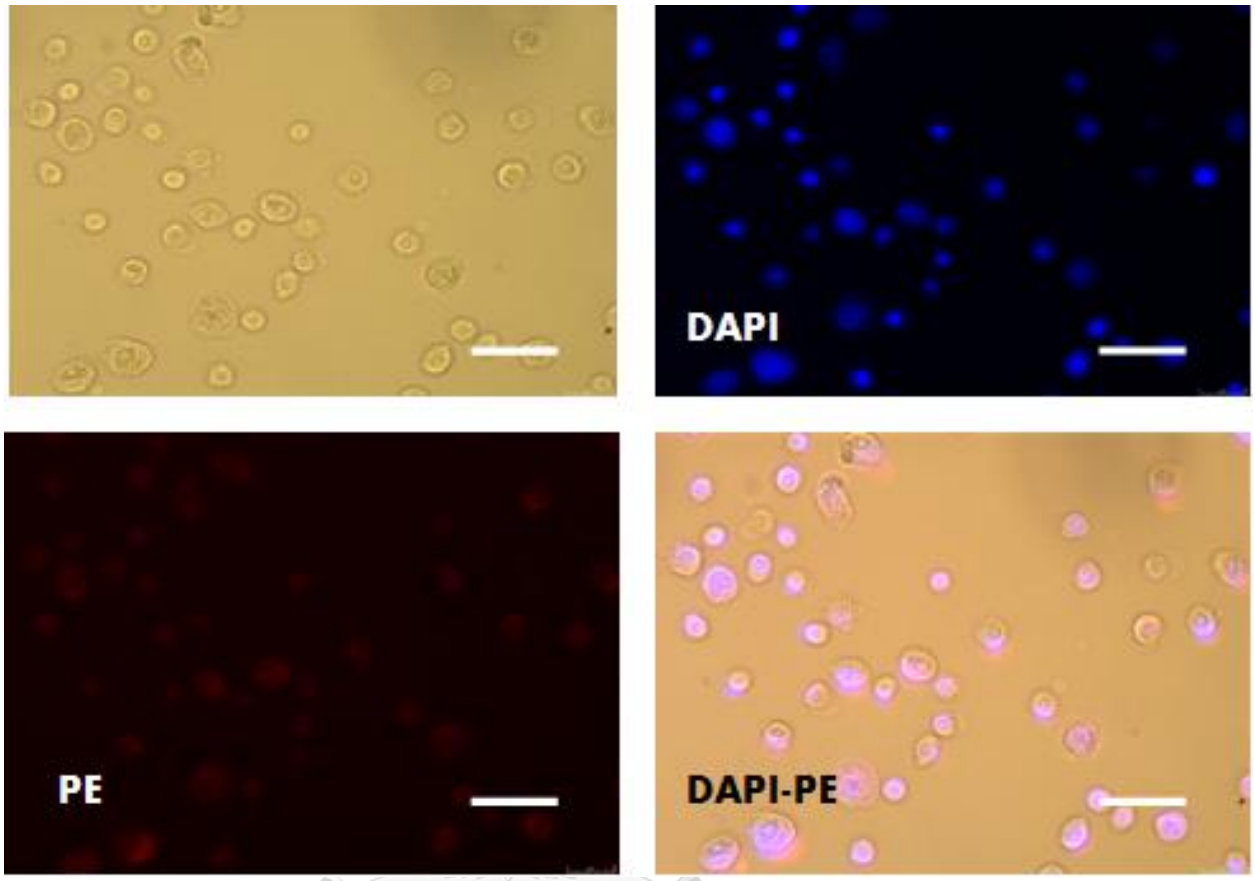


Figure 19. The Nanog protein detection in canine keratinocyte cell line.

The Nanog protein detection in canine keratinocyte cell line (Positive control). Fluorescence microscope, PE, Bar = 50 μm

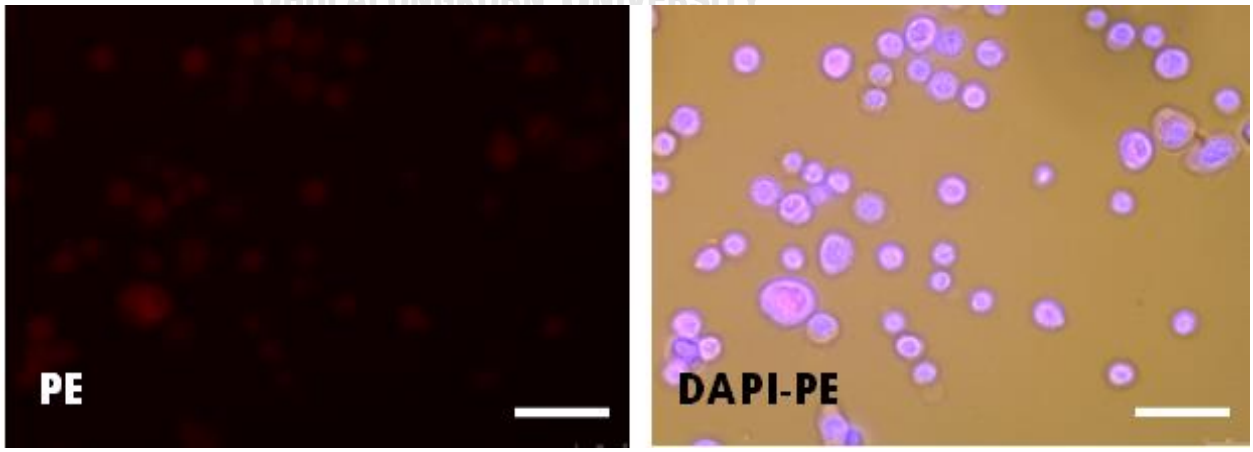


Figure 20. The Sox-2 protein detection in canine keratinocyte cell line.

The Sox-2 protein detection in canine keratinocyte cell line (Positive control). Fluorescence microscope, PE, Bar = 50 μm

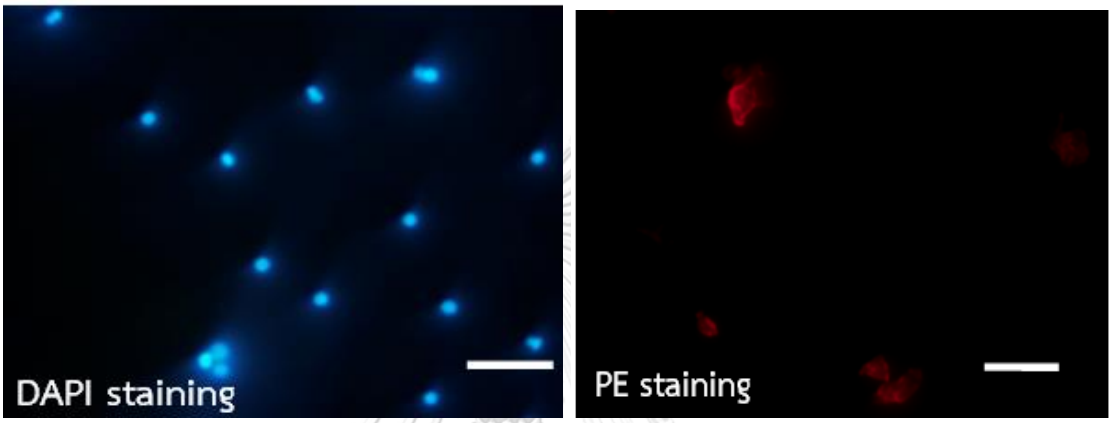


Figure 21. The Oct-4 protein detection in MCT sample.

Fluorescence microscope, PE, Bar =50 μm

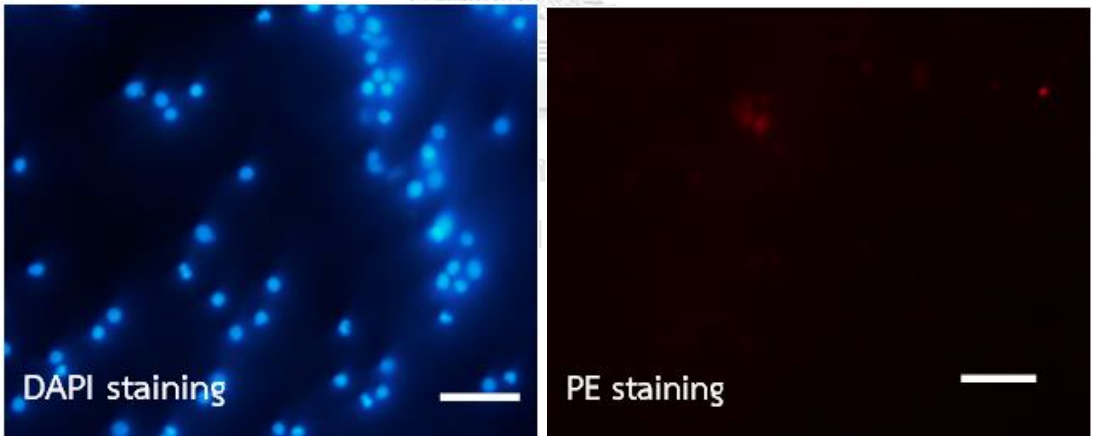


Figure 22. The Nanog protein detection in MCT sample.

Fluorescence microscope, PE, Bar =50 μm

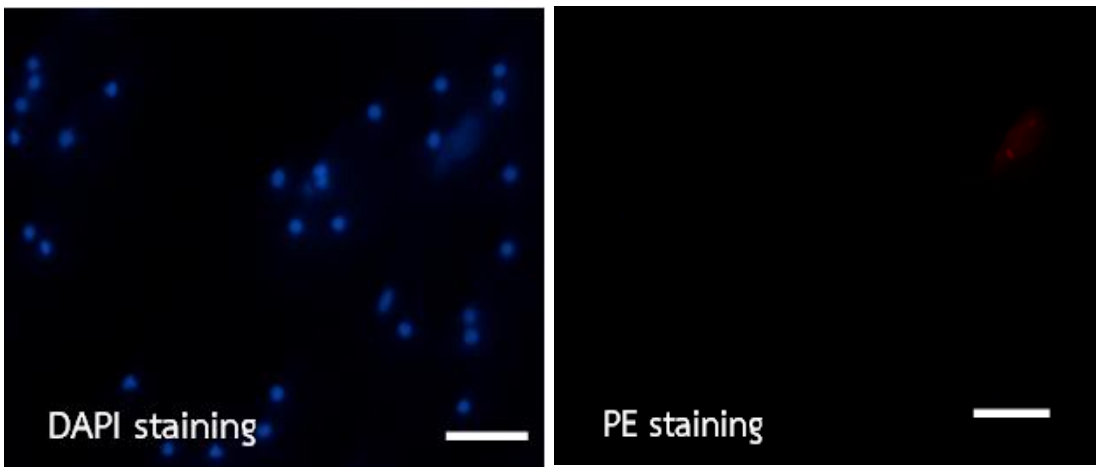


Figure 23. The Sox-2 protein detection in MCT sample.
Fluorescence microscope, PE, Bar =50 μm

5. The Expression of Transcription factor Oct-4, Nanog, and Sox-2 by Western blot technique.

The detected band of β-actin positive control was approximately 42 kDa. The results of western blot technique showed the expression of Oct-4, Nanog, and Sox-2 protein with low reaction intensity. The Oct-4 showed 1 band at 43 kDa, Nanog, 3 bands at 45, 35 and 25 kDa, approximately and Sox-2, 4 bands, at 77,65,35 and 26 kDa approximately when compared to the Canine keratinocyte cell line.

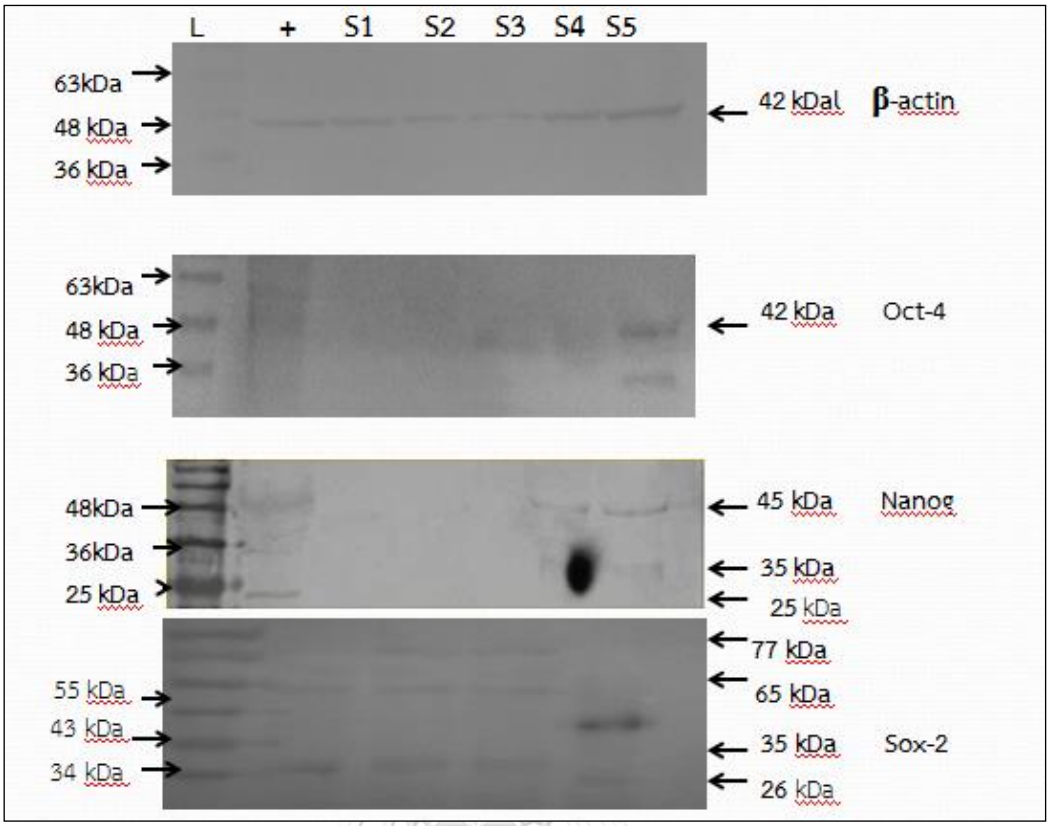


Figure 24. Western blot analysis.

The Oct-4, Nanog, and Sox-2 protein expression in MCT. The keratinocyte cell line was positive control. The expression of β-actin was used as a loading control.

L= protein ladder, + = positive control (Canine keratinocyte cell line), S1-S5=MCT samples

6.The Potential of the Detection of Transcription factor Oct-4, Nanog, and Sox-2 by Surface Plasmon resonance

The 10 MCT samples were determined transcription factor Oct-4, Nanog, and Sox-2 proteins by Surface Plasmon resonance. The SPR sensor was used a self-assembled monolayer (SAM) and investigated efficiency in water and phosphate buffered saline before detected antibody – antigen interaction. The result showed in Figure 25. The base line (at 60 min) was elevated at 120 min when MCT sample protein flow through the gold surface.

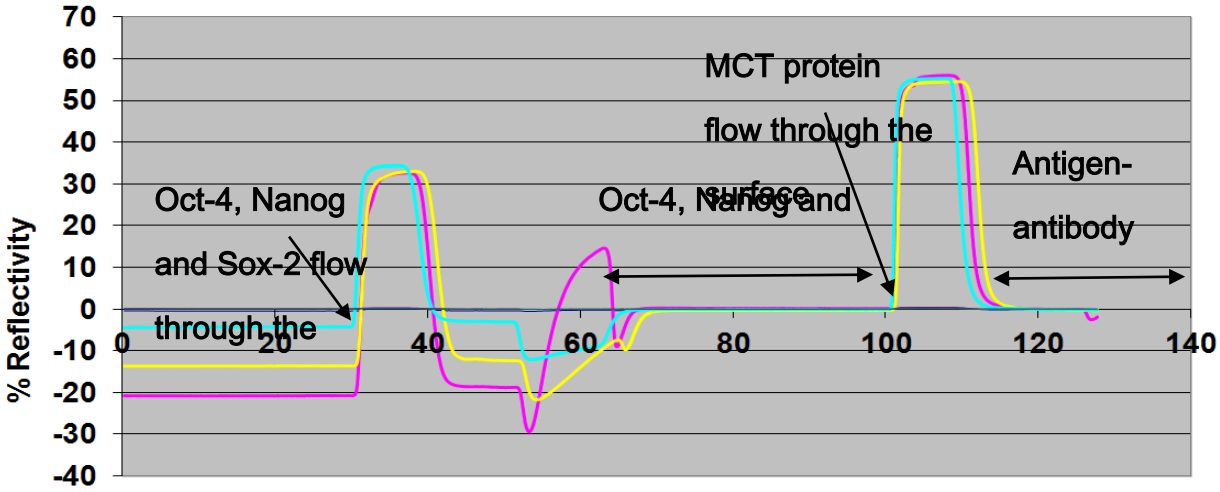


Figure 25. Graph of the expression of Oct-4, Nanog, and Sox-2 in MCT cases by SPR technique.

The sensorgram of anti-Oct-4, Nanog, and Sox-2 antibody . At 20 min: Oct-4, Nanog, and Sox-2 antibody flow through surface, at 120 min: antigen-antibody binding.(Blue graph=Oct-4 antibody, pink graph=Nanog antibody, yellow=Sox-2 antibody)

CHAPTER V

DISCUSSION AND CONCLUSION

MCTs are common and frequently malignant skin tumor in dogs. The mean age of dog affected MCT in this study is about 9 years old that is similar to the previous studies (Welle et al., 2008; Leidinger et al., 2014). This study showed that there were a variation in size and location of tumors that are also corresponded to the another studies, which suggested that sex of dog affected with MCT had no relationship with the incidence (Welle et al., 2008; Leidinger et al., 2014). MCT samples in this experiment were clinically recurrence tumors and had been classified in different grades by both Patnaik's and Two-tier histopathology grading system (Patnaik et al., 1984; Kiupel et al., 2011). This could be associated with the characteristic of MCT that has high incidence of recurrences (Webster et al., 2007a). In human, regards to the histopathological system of MCT, high grade MCTs have recently reported that a poor prognosis with short survival (Willmann et al., 2019). Which are not agreed in this studied. Our sample, the MCT cases were classified in low grade more than high grade and grade I, II more than grade III, with all of them are clinically recurrence cases.

Since, all of MCT samples were clinically recurrence after treatment which could be related with the other medical reports that have high incidence of recurrence after chemo-radiotherapy such as human rectal cancer with expression of major transcription factors *Oct-4* and *Sox-2*. Moreover, Lin and colleagues explained that *Oct-4* and *Sox-2* are the essential transcription factors that can induce tumor metastasis and chemo-resistance in colon cancer tumor (Lin et al., 2012).

It is suggested that RT-PCR technique could be detected the expression of transcription factor *Oct-4* and *Sox-2* genes in all MCT samples (100%; 30/30). The experiment also implied that

the MCT samples possessed the existent of pluripotency property. There are various medical reports regarding tumors having putative cancer stem cells have high tendency of recurrence (Clarke et al., 2006; Nguyen et al., 2012; Oskarsson et al., 2014; Plaks et al., 2015). Thus, the grading system cannot predicted the progression, metastasis and the survival time of MCTs because the MCTs have varied the biological behavior (Vargas et al., 2015).

Many previous results showed that expression of *Oct-4* and *Sox-2* may be associated with tumor relapse (Wang and Dai, 2010; Ferletta et al., 2011; Leis et al., 2012; Amaya and Bryan, 2015; Pitynski et al., 2015; Wilson-Robles et al., 2015). The *Oct-4* have the extra multi-function for cancer development such as blocks progenitor-cell differentiation, promotes tumorigenesis and inhibits apoptosis, so the pattern of co-expression can change the cellular normal function (Hochedlinger et al., 2005; Wang et al., 2013). The *Oct-4* and *Sox-2* were major transcription factors that co-operated to regulate the self-renewal and pluripotent of embryonic stem cell and expressed in various tumors such as oral squamous cell carcinoma, urinary bladder cancer, seminoma, prostate cancer human breast cancer, human vascular tumor, canine mammary gland tumor canine melanoma and canine adenocarcinoma (Wang and Dai, 2010; Ferletta et al., 2011; Leis et al., 2012; Amaya and Bryan, 2015; Pitynski et al., 2015; Wilson-Robles et al., 2015). In human cancers, the pattern of *Oct-4* and *Sox-2* expression, the stage and the progression of tumor could be predicted, such as over expression of *Oct-4* and loss of *Sox-2* expression associated with poor prognosis in human cervical cancer (Kim et al., 2015). Both *Oct-4* and *Sox-2* showed up regulated expression in many cancers such as oral squamous cell carcinoma, urinary bladder cancer, seminoma, prostate cancer, and breast cancer associated with poor prognosis (Atlasi et al., 2007; Gu et al., 2007; Ben-Porath et al., 2008; Chiou et al., 2008; Wang and Dai,

2010). Additionally, the *Oct-4* and *Sox-2* co-expression was found in the early stage of tumor such as in endometrial adenocarcinoma (Patnaik et al., 1984).

Since, all the MCT samples, the result of qRT-PCR demonstrated by the delta CT (Delta CT corresponding to the difference between target gene and reference gene) and the β- actin was used as internal control. The *Oct-4* expression in grade III and high grade tended to be higher than grade I, II and low grade. *Sox-2* expression, in grade III (Patnaik's system) and high grade (Two-tier grading system) tended to be lower than grade I, II, and low grade respectively, when compared to internal control. It is revealed that the *Oct-4* and *Sox-2* expression are not in parallel to the histopathology grade, they are opposition expressed in MCT cases.

In this study, all of 30 MCT samples also expressed both transcription factors *Oct-4* and *Sox-2* genes at mRNA level by using RT-PCR technique. While, the *Nanog* expressed only in positive control (keratinocyte cell line). There were no MCT samples expressing the *Nanog* gene that might be due to the unstable and short half-life of Nanog (Ramakrishna et al., 2011).

The *c-Kit* mutation in MCT was reported by Webster and colleagues (2006) and suggested that MCTs with *c-Kit* mutations resulting on high incidence of recurrence and death. However, the results in this study showed that all MCT samples did not have the *c-Kit* mutation in exon-11 while, all the samples were recurrence with expression *Oct-4* and *Sox-2.* Thus, there was no relation on the *c-Kit* mutation and the expression of *Oct-4* and *Sox-2* gene in MCT in this study.

In case of IHC study, the 30 MCT samples were conducted on the immunohistochemical expression of Oct-4, Nanog, and Sox-2 proteins. There were obviously positive expression of Oct-4, Nanog, and Sox-2 in all the MCT samples, at 90% (27/30), 80 % (24/30) and 80% (24/30) respectively. The positive staining cells of Oct-4 and Nanog were not difference in all grades ($p \geq 0.05$). Interestingly, the positive cells of Sox-2 in high grade was higher than low grade of MCT

samples with statistically difference ($p<0.05$). However, when compared to the pattern of expression of Sox-2 in MCT Patnaik's grading system.

Our findings indicated that the MCTs samples expressed major transcription factors Oct-4, Nanog, and Sox-2 proteins which agreed to the previous studies (Webster et al., 2007b; Joselevitch et al., 2018). In normal embryonic stem cell, the Oct-4 and Sox-2 prior work together and stimulate Nanog expression later. In this study the expression of Oct-4 and Sox-2 were correlated that similarly to the regulation pathway of Oct-4 and Sox-2 (Tai et al., 2005; Rizzino, 2013) which several studies employed the Oct-4, Nanog, and Sox-2 expression on prediction of the stage of cancer.

The MCT cases were demonstrated the expression of Oct-4, Nanog, and Sox-2 protein by Intranuclear Immunocytofluorescence. The results showed the localization of Oct-4, Nanog, and Sox-2 proteins in MCT cells. This technique can support the result of immunohistochemical staining pattern by the results of intranuclear and intracellular localizing Oct-4, Nanog, and Sox-2 proteins in MCT cells (Whitworth et al., 2014). In this regards, the nonspecific staining should be also considered for the IHC detection.

In case of protein detection, the results of western blot technique showed the expression of Oct-4, Nanog, and Sox-2 proteins in all grade of MCT samples. Canine keratinocyte cell lines was subsequently used as positive control in this experiment because of their self-renewal and pluripotency properties which expressed Oct-4 and Sox-*2* proteins (Tai et al., 2005). The results of western blot technique showed the Oct-4, Nanog, and Sox-2 protein with low reaction intensity of proteins. Which could be explained due to low number of positive cells in the MCT.

The molecular weight of Oct-4 protein was demonstrated, 1 band at 43 kDa which agreed to the previous report in human (Gatti et al., 2016). While, size of Sox-2 varied depending on the post- translational modification (Stevanovic et al., 1994). The Sox-2 protein were found, 4 bands at 77, 65, 35 and 26 approximately. Which is difference from previous researches in human, showed predicted band at 34 kDa or 36 kDa (Pan and Thomson, 2007). However, the result is in parallell to the molecular weight of canine Sox-2 which was calculated from the Sequence Manipulation Suite program (2000), at 77.68 kDa. Furthermore, it has shown that more than one band of the expected Sox-2. That could be due to *Sox-2* containing several spliced transcript protein variants (Shahryari et al., 2014). While, the bands of Nanog protein in this experiment were poor intensity due to the unstable property (Ramakrishna et al., 2011). The predicted band of Nanog were 42 kDa (human nanog). However, the study obtained 3 bands of Nanog at 45, 35 and 26 approximately. That was not related with the size of Nanog protein in canine embryonic stem cell (Hayes et al., 2008). However, the positive signal of Oct-4, Nanog and Sox-2 proteins in MCT cases have less signal that might be due to the low amount of the protein, limitation of western blot technique and non specific of antibody as well (Bass et al., 2017).

This study attempted to develop the Surface plasmon resonance (SPR) detecting the protein Oct-4, Nanog and Sox-2 with positive results. The advantage of this technique are high sensitivity and ability to measure the molecular interactions without labelling reagents (Nguyen et al., 2015). It is suggested the SPR technique is a potential tool for detecting protein expression together with the other methods, Western blot, IHC and IF. However, the limitation of SPR are the high sensitivity that leaded to the false positive. The further study, the non specific protein should be carefully further investigated.

Conclusion of the study

This study reveals that:

1. The MCT samples were clinically recurrent cases with various grades classified by Patnaik's system and two-Tier system.
2. The MCT samples did not demonstrate the c-kit mutation at exon-11.
3. The MCT tissues expressed the major embryonic transcription factors; Oct-4 and Sox-2 in mRNA level.
4. MCT grade, revealed that the *Oct-4* gene in high grade tended to be higher than low grade parallelly in grade III also tended to be higher than grade I and II respectively. On the contrary, *Sox-2* expression, in grade III and high grade tended to be lower than grade I and low grade respectively, when compared to internal control.
5. The MCT tissues were immunohistochemical expressed the major embryonic transcription factors; Oct- Nanog and Sox-2 at approximately of 80 % of all the samples.
6. Interestingly, the positive cells of Sox-2 in high grade was higher than low grade and grade III was also higher than grade I, II with statistically difference ($p<0.05$).
7. The MCT cells were intranuclear immunocytofluorescence localized the major embryonic transcription factors; Oct-4, Nanog and Sox-2 which in compatible to immunohistochemical staining patterns.
8. The western blot technic was weakly demonstrated the evidence of the major embryonic transcription factors; Oct-4, Nanog and Sox-2 protein with relatively molecular weight in the same pattern of the positive control.

9. The SPR technic is potentially used for detecting the the major embryonic transcription factors; Oct-4, Nanog and Sox-2 protein with high sensitivity and the non-specific problem should be further investigated.

By the obtained results that the major embryonic transcription factors; Oct-4, Nanog and Sox-2 expression both genes and protein could be demonstrated in MCT tissues.

Further studies

1. The further study on the transcription factors; Sox-2 which has played an interesting role in the MCT recurrence cases, should be investigated.
2. The others method; IHC, IF, western blot for the detection in protein level of the major transcription factors; *Oct-4, Nanog and Sox-2* could support in-part on the presence of pluripotency property in MCT tissue. That the limitation of these methods should be concerned.
3. The SPR is a potential technique for the protein detection in the Lab on chip model, that the further study should be developed.

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APPENDIX

Case No.	Two tier grading	Patnaik's grading	RT-PCR			qRT-PCR			IHC			Western blot		
			Oct-4	*Nanog*	*Sox-2*	*Oct-4*	*Nanog*	*Sox-2*	Oct-4	Nanog	Sox-2	Oct-4	Nanog	Sox-2
1	L1	Grade I	+	-	+	+	-	+	+	-	+	-	-	+
2	L2	Grade I	+	-	+	+	-	+	+	+	-	-	-	+
3	L3	Grade I	+	-	+	+	-	+	+	+	+	-	-	+
4	L4	Grade I	+	-	+	+	-	+	+	+	+	+	+	+
5	L5	Grade I	+	-	+	+	-	+	+	+	+	+	+	+
6	L6	Grade I	+	-	+	+	-	+	+	+	+	+	+	+
7	L7	Grade I	+	-	+	+	-	+	+	+	-	+	+	+
8	L8	Grade I	+	-	+	+	-	+	+	+	+	+	+	+
9	L9	Grade I	+	-	+	+	-	+	+	+	-	+	+	+
10	L10	Grade I	+	-	+	+	-	+	+	+	+	+	+	+
11	L11	Grade I	+	-	+	+	-	+	+	+	+	+	+	+
12	L12	Grade I	+	-	+	+	-	+	+	+	+	+	-	-
13	L13	Grade II	+	-	+	+	-	+	+	+	-	+	+	+
14	L14	Grade II	+	-	+	+	-	+	+	+	+	+	+	+
15	L15	Grade II	+	-	+	+	-	+	+	-	+	+	+	+
16	L16	Grade II	+	-	+	+	-	+	+	-	+	-	-	+
17	L17	Grade II	+	-	+	+	-	+	+	-	+	-	-	-
18	L18	Grade II	+	-	+	+	-	+	+	+	+	-	-	-
19	L19	Grade II	+	-	+	+	-	+	+	-	+	-	-	-
20	L20	Grade II	+	-	+	+	-	+	-	-	+	-	-	-
21	H1	Grade III	+	-	+	+	-	+	-	+	-	+	+	+
22	H2	Grade III	+	-	+	+	-	+	+	+	+	+	-	+
23	H3	Grade III	+	-	+	+	-	+	+	+	+	-	-	-

Case No.	Two tier grading	Patnaik's grading	RT-PCR			qRT-PCR			IHC			Western blot		
			Oct-4	*Nanog*	*Sox-2*	*Oct-4*	*Nanog*	*Sox-2*	Oct-4	Nanog	Sox-2	Oct-4	Nanog	Sox-2
24	H4	Grade III	+	-	+	+	-	+	+	+	-	-	-	-
25	H5	Grade III	+	-	+	+	-	+	+	+	+	+	+	+
26	H6	Grade III	+	-	+	+	-	+	+	+	+	+	+	+
27	H7	Grade III	+	-	+	+	-	+	+	+	+	+	+	+
28	H8	Grade III	+	-	+	+	-	+	-	+	+	-	-	-
29	H9	Grade III	+	-	+	+	-	+	+	+	+	-	-	-
30	H10	Grade III	+	-	+	+	-	+	+	+	+	-	-	-

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1. S. Meesuwan,D.Ketpun, A.Kaneungthong, P.Theewasutrakul,P. Piyaviriyakul , A. Sailasuta. A Preliminary Report on The Expression of Sox-2 in Canine Cutaneous Mast Cell Tumor-Putative Cancer Stem Cells. Proceedings of the 14th Chulalongkorn University Veterinary Conference CUVC 2015: Responsible for Lives April 20-22, 2015, Bangkok, Thailand

2. S. Meesuwan,D.Ketpun, A.Kaneungthong, P.Theewasutrakul,P. Piyaviriyakul , A. Sailasuta Expression Sox-2 Embryonic Transcription Factor of Putative Cancer Stem Cells in Canine Cutaneous Mast Cell Tumor. 32th World Veterinary Congress (WVC).2015.Istanbul, Turkey

3. S. Meesuwa, A. Sailasuta , K. Rattanapinyopituk, P.Theewasutrakul, D.Ketpun, A.Kaneungthong, P. Piyaviriyakul. Co-expression of Oct-4, Nanog and Sox-2 in Canine Cutaneous Mast Cell Tumor determined by RT-qPCR .Proceeding of the 15th Chulalongkorn University Veterinary Congress, April 20-22, 2016 Bangkok, Thailand.

4. S. Meesuwan, A. Sailasuta, K. Rattanapinyopituk, P. Piyaviriyakul.The Transcription factors Oct-4 and Sox-2 Proteins determination in Canine Cutaneous Mast Cell Tumors by Western Blot Technique. Proceeding of the 16th Chulalongkorn University Veterinary Congress, March 22-24, 2017 Bangkok, Thailand.

5. Sirilak Meesuwan, Prapruddee Piyaviriyakul, Kasem Rattanapinyopituk1and Achariya Sailasuta .CO-EXPRESSION OF EMBRYONIC TRANSCRIPTION FACTORS OCT-4 AND SOX-2 GENES IN RECURRENT CANINE CUTANEOUS MAST CELL TUMORS. Asian Veterinary Pathologists Congress, November 9-11, 2017 Karnataka, India.

6. S. Meesuwan, A. Sailasuta, K. Rattanapinyopituk, P. Piyaviriyakul Detection on the Embryonic transcription factors Oct-4, Nanog, and Sox-2 Proteins in Canine Cutaneous Mast Cell Tumors by Immunocytofluorescence technique. Proceeding of the 17th Chulalongkorn University Veterinary Congress, April 25-27,2018 Nonthaburi, Thailand.

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