การแสดงออกของอินโวลูครินในเซลล์เพาะเลี้ยงแบบสามมิติกับปัจจัยการพยากรณ์โรคมะเร็งผิวหนังชนิดสแควมัสเซลล์คาร์ซิโนมาในสุนัขป่วย และในเซลล์เพาะเลี้ยงที่เหนี่ยวนำด้วยแสงอัลตร้าไวโอเลต

นางสาวนงนุช อัศววงศ์เกษม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

INVOLUCRIN EXPRESSION IN THREE-DIMENTIONAL CELL CULTURE AND PROGNOSTIC PARAMETERS OF CANINE CUTANEOUS SQUAMOUS CELL CARCINOMA (SCC) IN CANINE PATIENTS AND IN ULTRAVIOLET RADIATION INDUCED CULTURED CELLS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
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Thesis Title	INVOLUCRIN EXPRESSION IN THREE-DIMENTIONAL CELL CULTURE AND PROGNOSTIC PARAMETERS OF CANINE CUTANEOUS SQUAMOUS CELL CARCINOMA (SCC) IN CANINE PATIENTS AND IN ULTRAVIOLET RADIATION INDUCED CULTURED CELLS
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นงนุช อัศวางศ์เกษม : การแสดงออกของอินโวลูครินในเซลล์เพาะเลี้ยงแบบสามมิติกับปัจจัยการพยากรณ์โรคมะเร็งผิวหนังชนิดสแควมัสเซลล์คาร์ซิโนมาในสุนัขป่วย และในเซลล์เพาะเลี้ยงที่เหนี่ยวนำด้วยแสงอัลตร้าไวโอเลต (INVOLUCRIN EXPRESSION IN THREE-DIMENTIONAL CELL CULTURE AND PROGNOSTIC PARAMETERS OF CANINE CUTANEOUS SQUAMOUS CELL CARCINOMA (SCC) IN CANINE PATIENTS AND IN ULTRAVIOLET RADIATION INDUCED CULTURED CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. สพ.ญ. ดร.อัจฉริยา ไศละสูต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. สพ.ญ. ดร.สมพร เตชะงามสุวรรณ, ผศ. น.สพ. ดร.ประพฤติดี ปิยะวิริยะกุล, 86 หน้า.

การศึกษานี้มีวัตถุประสงค์ดังนี้ 1) ศึกษาการแสดงออกของอินโวลูคริน (INV) และปัจจัยการพยากรณ์โรคมะเร็งผิวหนังชนิดสแควมัสเซลล์คาร์ซิโนมา (CSCC) 2) เพื่อพัฒนาการเพาะเลี้ยงผิวหนังชั้นกำพร้าจำลองของสุนัข และ 3) ศึกษาผลการแสดงออกของอินโวลูครินต่อกระบวนการเกิด CSCC ที่เหนี่ยวนำด้วยแสงอัลตร้าไวโอเลต โดยเริ่มจากการศึกษาความแตกต่างของการแสดงออกของโปรตีน ด้วยเทคนิคอิมมูโนฟลูโอเรสเซนต์ และ mRNA ของ INV, ไซโตเคราติน 10 (CK10) Ki67 และ p53 ในผิวหนังสุนัขปกติ (n=6) และ ในสุนัขป่วยด้วย CSCC (n=6) ในผิวหนังสุนัขปกติ พบการแสดงออกของโปรตีน INV และ CK10 ในไซโตพลาสซึม Ki67 พบในนิวเคลียสในชั้นหนังกำพร้าที่มีนิวเคลียส แต่ไม่พบการแสดงออกของโปรตีน p53 ปริมาณการแสดงออกของ mRNA ของ INV, CK10, Ki67, p53 ในสุนัขปกติและ ในสุนัขป่วยด้วยCSCC สอดคล้องกับผลการแสดงออกของโปรตีน พบว่า INV ในผิวหนังปกติมีปริมาณที่มากกว่า และ Ki67 มีปริมาณที่น้อยกว่า CSCC อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ไม่พบการแสดงออกของไวรัสชนิดแพพพิลโลมาใน CSCC ทุกตัวอย่าง และพบการแสดงออก p53 ชนิด wild type ใน CSCC มีแนวโน้มมากกว่าผิวหนังปกติ การพัฒนาเซลล์ผิวหนังชั้นหนังกำพร้าจำลองของสุนัขโดยการเพาะเลี้ยงเซลล์ไลน์ชนิด CPEK (canine progenitor epidermal keratinocyte cell line (CELLnTEC Advanced Cell Systems, Switzerland) แบบ 3 มิติจากด้วยเทคนิค air-liquid interface พบว่าเซลล์เพาะเลี้ยงแบบ 3 มิติ เกิดการพัฒนาคล้ายกับชั้นกำพร้าของผิวหนังสุนัขในวันที่ 14 และมีการแสดงออกของโปรตีนด้วยเทคนิคอิมมูโนฟลูโอเรสเซนต์ ของ INV และ CK10 แต่ไม่พบ Ki67 และ p53 ปริมาณการแสดงออกของ mRNA ของ INV และ CK10 ในเซลล์เพาะเลี้ยงแบบ 3 มิติ ไม่แตกต่างจากผิวหนังสุนัขปกติอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) และไม่พบการแสดงออกของ p53 และ Ki67 อย่างไรก็ตามไม่สามารถเพาะเลี้ยงเซลล์ปฐมภูมิจากผิวหนังสุนัขปกติ และ CSCC ได้ ศึกษาการยับยั้งการแสดงออกของยีน INV ในเซลล์ไลน์ CPEK สุนัขด้วย siRNA โดยออกแบบด้วยโปรแกรมออนไลน์ (https://us.bioneer.com/sirna/custom-sirna-ex.aspx) ได้คัดเลือกทดสอบจำนวน 3 สาย ได้แก่ INV-1, INV-2 และ INV-3 ผลการทดสอบคัดเลือกได้ INV-1 ที่สามารถยับยั้งการแสดงออกของ mRNA ของ INV ใน CPEK ได้ดีที่สุด ผลการไตเตรทปริมาณพบว่า 300 pmol ของ INV-1 เป็นปริมาณที่เหมาะสมและมีประสิทธิภาพ และเกิดความเป็นพิษต่อเซลล์น้อยที่สุด โดยเซลล์ CPEK ที่ทรานสเฟคชั่นด้วย siRNA ชนิด INV-1 ลงไป พบว่าเริ่มมีปริมาณการแสดงออก mRNA ของ INV ลดลงอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ตั้งแต่ชั่วโมงที่ 0 ของการเก็บเซลล์หลังผ่านขบวนการทรานสเฟคชั่น 5 ชั่วโมง และเริ่มกลับมาแสดงออกอีกครั้งใน 48 ชั่วโมง ทำการทดสอบเซลล์ไลน์ CPEK เพาะเลี้ยงภายใต้แสงอัลตร้าไวโอเลตชนิดบี ที่ 300 mJ/cm^2 พบการตายและลอกหลุดของเซลล์ CPEK ในทุกกลุ่มที่ได้รับแสงอัลตร้าไวโอเลตชนิดบี และพบปริมาณการแสดงออกของ mRNA ของ p53 เพิ่มขึ้นในทั้งสองกลุ่มที่ทำและไม่ทำการทรานสเฟคชั่นด้วย siRNA และพบว่ามีแนวโน้มเพิ่มขึ้นเมื่อได้รับแสงอัลตร้าไวโอเลตชนิดบีนานขึ้น โดยกลุ่มที่ได้รับแสงอัลตร้าไวโอเลตชนิดบี พบว่ามีปริมาณการแสดงออกของ mRNA ของ p53 มากกว่ากลุ่มที่ไม่ได้รับแสง อย่างมีนัยสำคัญทางสถิติที่ 24 ชม ($p<0.05$) ไม่มีความแตกต่างของการแสดงออก mRNA ของ INV, CK10, และ Ki67

ผลการศึกษานี้แสดงให้เห็นว่า การแสดงออกโปรตีนและmRNAของอินโวลูครินในสุนัขผิวหนังปกติและใน CSCCมีความแตกต่างกัน และสามารถพัฒนาวิธีการเพาะเลี้ยงผิวหนังชั้นกำพร้าจำลองของสุนัขได้โดยวิธีการเพาะเลี้ยงเซลล์ไลน์ชนิด CPEK (canine progenitor epidermal keratinocyte cell line) แบบ 3 มิติจาก ด้วยเทคนิค air-liquid interface อีกทั้งเป็นรายงานแรกของการใช้ siRNA ที่ออกแบบขึ้นยับยั้งการแสดงออกของ mRNA ของอินโวลูครินในเซลล์ผิวหนังชั้นกำพร้าในสุนัขได้นาน 48 ชั่วโมง สรุปได้ว่าการแสดงออกของ mRNA ของ INV ไม่มีผลต่อเซลล์เพาะเลี้ยงชนิดเคราติโนไซต์ที่เหนี่ยวนำด้วยแสงอัลตร้าไวโอเลตชนิดบีที่ 300 mJ/cm^2 ที่เป็นสาเหตุของการเกิดมะเร็งผิวหนังชนิดสแควมัสเซลล์คาร์ซิโนม่า

ภาควิชา พยาธิวิทยา
สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์
ปีการศึกษา 2560

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
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5575405831 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: CANINE KERATINOCYTE / THREE DIMENSIONAL CELL CULTURE / SIRNA / UV RADIATION

NONGNUT ASSAWAWONGKASEM: INVOLUCRIN EXPRESSION IN THREE-DIMENTIONAL CELL CULTURE AND PROGNOSTIC PARAMETERS OF CANINE CUTANEOUS SQUAMOUS CELL CARCINOMA (SCC) IN CANINE PATIENTS AND IN ULTRAVIOLET RADIATION INDUCED CULTURED CELLS. ADVISOR: PROF. DR.ACHARIYA SAILASUTA, CO-ADVISOR: ASSOC. PROF. DR.SOMPORN TECHANGAMSUWAN, ASST. PROF. DR.PRAPRUDDEE PIYAVIRIYAKUL, 86 pp.

The objectives of this study are 1) to demonstrate the involucrin (INV) expression and prognostic parameters in normal skin and cutaneous squamous cell carcinoma (CSCC), 2) to develop the artificial epidermis in dog and 3) to study the effect of INV expression on pathogenesis of ultraviolet radiation induced CSCC in dog.The first experiment is to determine the protein expression by Immunofluorescence technic (IFC) and mRNA expression of INV,Cytokeratin 10 (CK10), Ki67 and p53 normal skin(n=6) and CSCC (n=6) in dog.The protein expression pattern was demonstrated that INV and CK10 were positive in intra-cytoplasmic pattern in nucleated epidermis. While, intra-nuclear pattern was positive with Ki67 but was negative for p53. The levels of the mRNA expression of INV, CK10, Ki67, and p53 in the normal skin and CSCC in dog are in correspondence with IFC that INV of normal skin revealed more than CSCC, on the contraryKi67 in normal skin was less than CSCC with statistically difference (*p<0.05*). The papilloma virus was negative in all samples. Though, p53 has not shown any statistically difference (*p>0.05*), and the mRNA expression in CSCC tends to be more than in normal skin.The second experiment is the cultured commercial canine keratinocyte cell line (CPEK, C CELLnTEC Advanced Cell Systems, Switzerland) was successfully conducted by producing the 3 dimensional (3D) epidermal skin appearance under air-liquid interface technique. The 3D cell culture showed the epidermal characteristic similar to the epidermis on 14 days cultured. For IFC, the INV, CK10 revealed positive result while ki67 and p53 showed negative result. The mRNA expression, there were no statistical difference of INV, CK10 between 3D cultured CPEK and normal skin (*p>0.05*). The p53 and ki67 were negative in 3D cell cultured. In the same cultured condition, the primary cell culture both normal skin and CSCC could not be obtained good yields. The third experiment was designed siRNA for INV by online program ((https://us.bioneer.com/sirna/custom-sirna-ex.aspx), the 3 sets; INV-1, INV-2 and INV-3 could be obtained. The treatment of cultured CPEK with INV-1 siRNA for INV knockdown showed the best result among 3 sets in the reduction of mRNA levels. From the titration, 300 pmol demonstrated the highly effective with minimum cytoxicity to cultured cells. The mRNA expression of INV started to decrease with statistical difference (*p<0.05*) at 0 h after 5 h transfection and slightly increasing at 48 h. The cytologic appearance of cultured CPEK were initially observed after 300 mJ/cm^2 of UVB irradiation exposure, at 6 h. The cell showed a number of dead and sloughing cells in the siRNA transfected with UVB irradiation group in comparison to control group. The mRNA expression of wild type p53 of cultured cell was increased after 6 h exposure of 300 mJ/cm2 of UVB and gradually increased its expression when increasing exposure time respectively. After the cells were harvested at 24 h, the results of p53 mRNA expression were statistically difference increased in cultured cell both; with and without siRNA transfection under UVB irradiation groups more than without UVB irradiation groups (*p<0.05*). There was no statistically difference of INV, CK10, and Ki67 expression between with and without UVB irradiation groups.

The obtained results demonstrated that the protein expression by IFC and the level of mRNA expression of INV are difference between normal skin and SCC in dog. The 3D cultured CPEK could be developed as an artificial epidermis in dog. Finally, this is the first report on the design of siRNA transfected in the cultured CPEKfor INV's knockdown. It is suggested that INV doesn't effect on the induced in cultured keratinocyte by the UVB irradiation at 300 mJ/cm^2, which is the major cause of CSCC in dog.

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LIST OF ABBREVIATIONS

µl	microliter
µm	micrometer
3D	three-dimension
AP	activator protein
CE	cornfield envelope
CK	canine normal skin
CK10	cytokeratin 10
CPEK	commercial canine progenitor keratinocyte cell line
CSCC	Cutaneous squamous cell carcinoma
DAB	diaminobenzidine tertrahydrochoride
DMEM	Dulbeccos' Modified Eagles medium
dsRNA	double-stranded RNA
ESCC	esophageal SCC
FFPE	formalin-fixed paraffin-embedded (tissue)
h	hour
H&E	hematoxylin and eosin
IFC	Immunofluorescence
IgE	immunoglobulin E
igG	Immunoglubulin G
IHC	Immunohistochemistry
INV	Involucrin
L	liter
min	minute
miRNA	micro-RNAs
ml	milliliter

mm	millimeter
mRNA	messenger ribonucleic acid
NSCLC	non-small-cell lung carcinoma
OCT	optimum cutting temperature
PCR	polymerase chain reaction
PP	papilloma
qPCR	quantitative real-time PCR
RNAi	RNA interference
RT-PCR	reverse transcription PCR
SCC	Squamous cell carcinoma
sec	second
siRNA	Small interfering RNA
UV	Ultraviolet
UVR	Ultraviolet radiation

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Ultraviolet radiation (UVR) is well-known as the cause of skin cancer in humans and animals. Greenhouse gases crisis, trap more heat and the ozone layer allows more dangerous UV light to penetrate. Previous studies have shown that every one percent of ozone layer thins can raise three percent of human skin cancer (de Gruijl, 1999). Moreover, skin cancer is a common cancer found not only in humans but also in pets because pets share similar environment and life style to humans. Also, many spontaneous occurring cancers in pets have similar characteristics and behaviors to those in human counterpart. Therefore, this is why use companion animals are as a model to study human diseases. Especially, cutaneous squamous cell carcinoma (SCC) is caused by chronic UV exposure. In Thailand, more than half of the dogs and cats stay outdoor. From the record at Veterinary oncology clinic (The Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University) during 2010-2013, the skin tumors comprised 31.5% of all the diagnosed tumor cases and 22.5% of them is SCC (82.9% in dog, 17.1% in cat) and numerous of SCC cases were multi-recurrence and more progressive within 2-3 months after treatments because of late detection. The major risk factors of dog cutaneous SCC induce their less pigmented skin and short hair coats, similar characters to white or Caucasian people (Warland, 2011; Webb, 2009; Yan et al., 2011).

Cutaneous SCC is induced by various cellular pathways, such as p53 mutation, loss of apoptosis mechanism, resulting in development of genetic

instability and loss of cell cycle control. Changing of precursors and cellular signalings of cancerous cell relates to enhance their mitogenesis and uncontrolled proliferation (Melnikova and Ananthaswamy, 2005; Ratushny et al., 2012). Therefore, many precursors and signaling markers have been applied for the earliest clinical detection, cancer bio-behavioral investigation and cellular response to treatments (Chanvorachote et al., 2012). Involucrin (INV) is a cornified envelope precursor protein, which was discovered since 1977 (Eckert et al., 1997; Rice and Green, 1977) and previously uses as an early stage marker of human terminal keratinocyte differentiation (Rice and Green, 1977). Various studies have showed the INV efficacy in other aspects, such as indicator for wound re-epithelialization enhancement (Obedencio et al., 1999), marker for atopic lesion with reduction of cellular proliferation without apoptosis (Hertle et al., 1992; Jensen et al., 2004), premalignant lesion marker (Balasubramanian and Eckert, 2004; Caldwell et al., 1997; Li et al., 2000; Nozoe et al., 2006), and endogenous marker in hypoxia of squamous cell carcinoma (Chou et al., 2004). This relationship explains via Activator protein-1 (AP-1) transcription factor mechanism, which is an important regulatory proteins in epidermis and cell biological processes, such as keratinocyte differentiation, proliferation, apoptosis and transformation in cancer progression (Eckert et al., 2004; Rorke et al., 2010). However, use of INV for cancer prognosis in veterinary has been limited. The study was reveal an existence of INV for canine skin cancer, that useful for cancer diagnosis, prognosis and/or treatment in the future.

1.2 Studies of the thesis

This study is aimed to develop the use of INV on veterinary oncology field by investigating INV expression in canine cutaneous SCC by comparing between normal skin and cutaneous SCC. Moreover, 3-dimensional skin culture were developed for investigating an importance of INV in cutaneous SCC under experimental model.

1.3 Objectives

1) To compare the INV expressions and other cancer prognostic markers between normal skin and cutaneous SCC in dog in vivo and in vitro by immunofluorescence and q-PCR.
2) To develop the three dimensional model of cultured canine keratinocyte and cutaneous SCC, in order to investigate the effect of INV siRNA transfection following UVB irradiation.

1.4 Research questions

1) Does the INV expression change in canine cutaneous SCC?
2) Does the variation of INV expression in canine cutaneous SCC relate to other cancer prognostic markers?
3) Does the INV expression change involving the pathogenesis of canine cutaneous SCC induced by UVB irradiation?

1.5. Hypothesis

1) The INV expression changes in canine cutaneous SCC
2) The changed of INV expression relates to other cancer prognostic markers
3) The changed of INV expression involve the pathogenesis of canine cutaneous SCC induced by UVB irradiation

CHAPTER II

LITERATURE REVIEW

2.1 From keratinocyte to cancer

2.1.1 Epidermis and keratinocyte hierarchy

Importantly, epidermis is the first protective barrier against the external environmental pathogen or chemical exposure and fulfills special function for homeostasis and temperature control of the body. The epidermis is a thin nonvascular skin layer consisting mainly of stratified epithelial keratinocytes, that undergo continuous and rapid proliferation and progression from mitotic activity of primitive basal stem cells, to intermediate transit-amplifying (TA) cells, and differentiated superficial cells at the terminal differentiation process where they are sloughed or exfoliated at the outer surface (Eckert et al., 1997; Lippens et al., 2009; Nishifuji and Yoon, 2013; Raj et al., 2006).

Differentiation of keratinocytes has been subdivided into sublayers or strata of epidermis. The basal layer (stratum basale) is a monolayer of cuboidal cells attached to the basement membrane (lamina basalis) by hemidesmosomes. This layer contains proliferating stem cells that continuously provide the stratified epithelium with new cells. The intermediate spinous layer (stratum spinosum), the cells reinforce their cytoskeletal keratin filament network, and adjacent cells interact via many desmosomes, a specialized type of cell junction, to resist physical trauma. The granular layer (stratum granulosum), the keratinocytes become more flattened and express certain proteins, such as profilaggrin and loricrin, which aggregate to form the typical keratohyalin granules, as products of keratinocyte

differentiation. In addition, lipids are produced and stored in lamellar bodies (Eckert et al., 1997; Nishifuji and Yoon, 2013). An increase in intracellular Ca2+ activates transglutaminases, which cross-link different structural proteins beneath the plasma membrane to form cornified envelope (Eckert et al., 2004). The cornified layer (stratum lucidum and stratum corneum), the cells lose their nuclei and other organelles, proteins are cross-linked at the inner side of the cytoplasmic membrane to form a cornified envelope (CE), and the dead cells are connected by corneodesmosomes (modified desmosomes containing corneocyte-specific components such as corneodesmosin). Lipids are extruded to form a water-repelling envelope around the CE, thereby assuring adequate impermeability of the mammalian epidermis. Finally, the dead corneocytes or cornified envelopes are shed during desquamation (Li et al., 2000; Lippens et al., 2009; Nishifuji and Yoon, 2013; Steinert and Marekov, 1997). Achieving these morphological alterations relies on executing a preset program of differentiation that requires tight regulation of gene expression (Eckert et al., 2004; Nishifuji and Yoon, 2013; Raj et al., 2006).

2.1.2 Programmed cell death in balancing renewed keratinocytes

Programmed cell death (apoptosis) in normal keratinocytes is usually delayed until terminal differentiation. Apoptosis is a beneficial mechanism that regulates normal growth and development and hence self-renewal of tissue and early eliminate mutant keratinocytes that are transformed by carcinogens. The tumor suppressor protein p53 is an important molecule that activates and facilitates either cell cycle arrest to initiate excision repair or mediates apoptosis depending on the extent of damage. The p53-mediated cell death occurs through direct interactions with mitochondrion. Temporary UV-exposed

cells can induce apoptosis via activation of intrinsic/ mitochondria pathway and cell surface death receptors which cluster or multimerize (as in the case of CD95) and recruit adaptor proteins such as FADD (Fas-associated protein with death domain) resulting in the activation of the extrinsic caspase pathway as FADD recruits and activates caspase-8 (called Fas-Fas ligand interaction), which in turn cleaves and activates caspase-3. Then, reactive oxygen species (ROS) have been generated lipid peroxidation in cell membranes and cytochrome c release from the mitochondria into the cytosol. Cytochrome c forms a complex with Apaf-1 (apoptotic protease activating factor-1) leading to caspase-9 recruitment and activation. This schema is an over-simplification of the signaling pathways that leads to apoptosis and many other complexes interactions are known to exist. However, apoptosis mechanism is unstable by excessive or chronic carcinogen exposure, which can be induced multiple cascades of molecular signaling events which induce inflammation, immunosuppression, failure of apoptosis, and aberrant differentiation (Berman and Cockerell, 2013; de Gruijl, 1999; Guzman et al., 2003; Melnikova and Ananthaswamy, 2005; Ratushny et al., 2012; Yan et al., 2011).

2.1.3 Transformation of keratinocytes to squamous cell carcinoma

Generally, squamous cell carcinoma (SCC) is a malignant epithelial transformation that can occurs in several different anatomic sites covered with squamous epithelium. On skin SCC, malignant transformation of keratinocyte epithelium is classified cutaneous SCC or epidermoid carcinoma, is a common tumor involving the skin (Blackwood, 2011; Dobson, 2011; Norman, 2006; Vail, 2007; Webb, 2009). The genetic abnormalities responsible for the development of cutaneous SCC are incompletely understood even in human

oncology. In addition, several genetic changes in cutaneous SCC have been suggested to be of prognostic value in humans with this disease (Vail, 2007).

Risk factor and etiology

There was high cutaneous SCC incidence reported in lack skin pigment or white or sparse hair coat dogs similar to white or Caucasian people. Older dogs are the greatest risk (8-10 year). SCC is the second most common malignant neoplasm of the skin compared to the first mast cell tumor (Sailasuta, 2013; Webb, 2009). The common sites of cutaneous SCC are the nail bed, scrotum, nasal planum, legs, and anus. No sex predilection has been noted. However, digital SCC was overrepresented in dark hair coats and more invasive. Major risk factor of cutaneous SCC, similar to human, is induced by solar or UV radiation (DNA damaging agent), especially higher occur in those of lightly pigment, fair skinned or short coat than darker skin or long hair coat. For minor effect, papillomavirus DNA has been detected in 15% of SCC dogs and chronic dermatosis, which abnormalities in molecular and genetic events has already been discussed (Blackwood, 2011; Chandrashekaraiah et al., 2011; Warland, 2011; Webb, 2009).

Pathogenesis of human cutaneous SCC was purposed to drive from transformed keratinocyte mutant clones of immature or undifferentiated that cannot process to corneocytes after carcinogen activation. Acute effects of UV radiation are DNA damage and the peroxidation of lipids and protein crosslink, which lead to erythema, sunburn, and immunosuppression. However, apoptosis mechanism can occur through multiple overlapping and non-overlapping pathways, by trigger apoptosis via p53-dependent manner and Fas–Fas ligand pathways. A variety of DNA damaging agents were induce p53 tumor suppressor protein that leads to cell cycle arrest, allowing the cellular

DNA repair pathways to remove DNA lesions before the onset of DNA synthesis and mitosis. Unfortunately, exposure to chronic UV irradiation suspected in over DNA damage accumulation, acquisition of p53 mutation and loss of Fas–Fas ligand interaction lead to dysregulation of apoptosis, abnormal proliferation of mutant clones of keratinocytes that undergo clonal expansion and accumulate various gone mutations. However, in this step if patients have well immunity, mutant clone may be cleared. But if their immunity are suppressed, mutant clones were form to imprisoned clone or preneoplastic lesions (actinic keratosis; AK) or solar keratosis, and progress into squamous cell carcinoma (Di Girolamo, 2010; Guzman et al., 2003; Melnikova and Ananthaswamy, 2005; Ratushny et al., 2012).

There are a number studies evaluating p53 in canine cutaneous SCC. When p53 is present in wild-type form, its short half-life prevents detection of the protein with IHC. The presence of a detectable form of the p53 protein correlate with mutations within the coding sequence. The IHC studies of p53 in canine cutaneous SCC revealed that 29.5%, or 19 of 65 tumor studied, were positive of p53 overexpression (Murakami et al., 2000; Teifke and Lohr, 1996). Favrot et al. (2009) studies have raised questions as to the relative roles of papillomavirus infection and UV irradiation in the development of different subtypes of cutaneous SCC because both mechanism of tumorigenesis can result in increased identification of p53 (Favrot et al., 2009).

Gross and histologic descriptions

Pathologic lesions of cutaneous SCC in dog lesion often showed slow growing. Proliferative lesions may vary from a red, firm plaque to a cauliflower-like lesion that often ulcerates and progress to a more proliferative, raised tumor, locally invasive, regional lymph node spread may be occurred with

poorly differentiated but metastases are rare (Chandrashekaraiah et al., 2011; Warland, 2011; Webb, 2009). On cytology, it can be poorly differentiated SCC when consists of predominant immature cells, and well-differentiated tumors containing more mature cells. Criteria of malignancy include prominent anisocytosis and anisokaryosis, multinucleated cells, and variable numbers and sizes of nucleoli (Chandrashekaraiah et al., 2011; Webb, 2009). On histology, SCC is classified as grade 1-3 from overall differentiation, mitotic index, nuclear pleomorphism, invasion, stromal reaction (Ehrhart, 2007). The initial crusting lesions often represent carcinoma *in situ* or pre-invasive carcinoma (i.e., Tis clinical stage). Generally, squamous cell carcinomas involving the skin of the flank and ventral abdomen in the dog are locally invasive and have a low metastatic potential; however, multiple lesions often are present throughout the skin of the ventral abdomen, ranging from carcinoma in situ to more infiltrative and nodular SCC (Vail, 2007). The typical well-differentiated SCC maintains a loose epithelial maturation sequence. The neoplastic cells form irregular whorls and cords within the tumor. Nests of neoplastic cells are surrounded by stroma and may have the equivalence of the basal cell layer at the outer edge of the nest and the keratin-producing layer in the center, creating the classic appearance of intensely eosinophilic, densely packed rings of keratin (a keratin pearl). In poorly differentiated, epithelial layering is indistinct, cells are smaller, and keratinization is less likely to be seen, the signet ring SCC has been reported (Chandrashekaraiah et al., 2011). A histologic variant of SCC, so-called signet-ring SCC, also has been reported in the dog (Espinosa de los Monteros et al., 2003).

Clinically, the prognosis for cutaneous SCC in dogs is most strongly predicted by the tumor stage or tumor-node-metastasis (TNM) system as guide line of epidermal or dermal origin tumors (excluding lymphoma and

mastocytoma) that devised by the World Health Organization (WHO, 1998) as below (Vail, 2007);

T: Primary tumor

Tis Preinvasion carcinoma (carcinoma *in situ*)

T0 No evidence of tumor

T1 Tumor<2cm maximum diameter, superficial, or exophytic

T2 Tumor 2-5 cm maximum diameter, or with minimal invasion irrespective of size

T3 Tumor>5cm maximum diameter, or with invasion of the subcutis, irrespective of size

T4 Tumor invading other structure such as fascia muscle, bone, or cartilage

Tumor occurring simultaneously should have the actual number recorded. The tumor with the highest T category is selected and the number of tumor indicated in parentheses. Successive tumor should be classified independently.

N: Region lymph nodes (RLN)

N0 No evidence of RLN involvement

N1 Movable ipsilateral node

N1a Nodes not considered to contain growth

N1b Nodes considered to contain growth

N2 Movable contralateral or bilateral nodes

N2a Nodes not considered to contain growth

N2b Nodes considered to contain growth

N3 Fixed nodes

M: Distant metastasis

M0 No evident of distant metastasis

M1 Distant metastasis detected-specify site (s)

In addition, other factors that may be predictive are the histologic grade, proliferation rate, vascular endothelial growth factor (VEGF) expression, apoptotic rate, and nuclear morphometry; however, further investigation is required to establish the predictive power of these factors (Vail, 2007).

2.1.4 UV radiation induced SCC carcinogenesis

Radiation can be divided into 2 major categories. The first is ionizing radiation, produced by various isotope that emit either gamma rays or particulate radiation (e.g., alpha and beta particles), and the second category is UV radiation (Cullen, 2002).

UV radiation is divided into three spectra: UV-A, which has recently been shown to be carcinogenic in laboratory animal, UV-B, which is a well-known cause of cutaneous neoplasia; and UV-C, a potent mutagen that is efficiently filtered out by the earth's ozone layer before it reach the surface. Solar UV irradiation at the earth's surface is approximately 90–99% UVA and 1–10% UVB (Verschooten et al., 2006). However, UV-B radiation is the portion of the spectrum that is most involved in cutaneous neoplasia. It produces a characteristic mutation at sites in DNA where two pyrimidine bases (i.e. cytosine and thymine) are found together. The radiation produces a dimer of these molecules that can lead to mutation when they are repaired incorrectly (Cullen, 2002). The importance of this type of genetic injury in the pathogenesis of cancer is supported by the presence of mutations in the dipyrimidine regions of the ras oncogene and p53 tumor suppressor gene in both human and mice following UV-B exposure. The oncogenicity of UV-B radiation may be

augmented by its deleterious effect on immunity, which may interfere with the recognition and destruction of tumor cell by the immune system. In contrast to UV-B radiation, UV-A radiation is not efficiently absorbed by DNA and protein. UV-A may cause DNA damage indirectly through the formation of free radicals and active oxygen species (Cullen, 2002; Norman, 2006).

The majority of neoplasm that are induced by UV irradiation arise in the epidermis, site of maximal exposure. In human, UV exposure increases the risk of squamous cell carcinoma and basal cell carcinoma. Malignant melanoma may also be linked to UV exposure, but the evidence is less persuasive. Squamous cell carcinoma is associated with UV exposure in animals and these tumor overexpress p53 and Ki67 (Norman, 2006), but more data is needed to determine of other types of skin neoplasia are induced by UV radiation in animals (Cullen, 2002). Excessive UV stimulate suppressor T lymphocyte, and this may activate oncogenic viruses or directly suppress host antitumor activity (Norman, 2006).

Many experimental presented effect of UVA (320–400 nm) and UVB (290–320 nm) solar UV on in vitro experiments. The trials almost calculate cell UV exposed by energy or mJ/cm2, the energy various from 10-300 mJ/cm2 depend on subjective of each researches (Galus et al., 2009; Keller et al., 2007; Sesto et al., 2002; Zhang, 2006).

2.2 Tumor markers related SCC

2.2.1 General and useful markers

Although histopathology remains a standard conventional method used for SCC diagnosis but recent biochemical and physiological approach function and molecular characteristic of cancer are contributed as major breakthrough in early and accelerate diagnosis, prognosis and therapy (Berretta

and Moscato, 2010; Kumar and Pawaiya, 2010). Over a decade, tumor marker has evolved as an indispensable tool for cancer assessment. New tumor markers were identified by oncologists timely to improve prognosis and behavior of the tumor. Tumor markers can be measured quantitatively or qualitatively by antigen-antibody based techniques (ELISA-enzyme linked immunosorbant assay, radio-immunoassay, precipitin tests, flow-cytometry, immunohistochemistry, immunoscintigraphy) and molecular genetic methods. Numerous markers of human cancer have been applied on veterinary oncology field because several human and dog neoplasm show similar histopathology and clinical behavior characterization (Bongiovanni et al., 2009) but still have some questions and limited information for veterinary work.

Tumor markers for SCC help reflect staging of the disease and its prognosis. Many candidate markers were demonstrated useful by veterinary oncologists. Recent publications report many useful markers, which approved for both human and canine SCC study, for example, p53, a tumor suppressor genes regulating cell-cycle progression and cell survival. Positive p53 gene mutation indicates developing SCCs and other epithelial (Keller et al., 2007). MKI67 (Ki-67), proto-oncogene regulating cell proliferation, is cell proliferation index marker typically increased in tumors (Scholzen and Gerdes, 2000) and found related to rapid growth and recurrence in non-melanotic skin cancer (NMSC) (Martins et al., 2009; Nozoe et al., 2006) and poor prognosis in canine SCCs (Assawawongkasem et al., 2016). Cadherin-1 (CDH1), also known epithelial cadherin (E-cadherin), is a tumor suppressor gene. Down-regulation of CDH1 is linked to increased potential of tumor invasiveness and distant metastasis. The frequencies of CDH1 promoter hypermethylation appear to be correlated with advanced stage of cutaneous SCC in both dog and human (João et al., 2011). Nuclear survivin, a member of the inhibitor of apoptosis protein (IAP) family,

encoded by a complex gene and functions in both cell-division and anti-apoptotic pathways was reported increased expression in pre-neoplastic and neoplastic lesions of SCCs both in human and dogs (Bongiovanni et al., 2009). Matrix metalloproteinases (MMPs), a family of zinc-containing endopeptidases were strongly detected in multiple stages of cancer progression including the acquisition of invasive and metastatic properties. Such as, MMP-2, MT-MMP that shown association with invasive potential and poor outcome of human and canine cutaneous SCC (Nakaichi et al., 2007; Roh et al., 2012).

Many particular tumor markers used in human had limited application in canine SCC, for example, significant p63 expression in poorly differentiated non-melanotic skin cancer and routine use in prognosis of human SCC, however, it is not significant in canine cutaneous SCC (Gama et al., 2003). CCND1 (Cyclin D1), proto-oncogene regulating cell-cycle progression, acts by phosphorylating and inactivating the retinoblastoma protein. It is frequently over expressed in keratinocyte carcinogenesis. CCND1 is associated with poor prognosis in NMSC, esophageal SCC (ESCC), and non-small-cell lung carcinoma (NSCLC), but it is not correlated with development of canine cutaneous SCC (Murakami et al., 2000). The oncogene c-erbB-2, significant sequence homology with epidermal growth factor receptor (EGFR), belongs to the RTK superfamily acting as a central transducer of multiple signaling pathways. Its over expression has been associated with human SCCs but is not significant in dog (Rungsipipat et al., 2000). Cyclooxygenase-2 (COX-2) is an enzyme that functions in protein metabolism by increasing prostaglandin synthesis and plays a role in tumorigenesis. Up-regulation of COX-2 was shown in many cancers and has significant role in human cutaneous SCC, but is not significant in canine cutaneous SCC (Bardagi et al., 2012).

However, some tumor markers were positive in dog but not in human, such as vascular endothelial growth factor (VEGF), which is an important signal transduction protein involved in both vasculogenesis and angiogenesis. VEGF was identified that particularly striking in canine cutaneous SCC on the toe, but it is no significantly correlated with NMSC in human (Maiolino et al., 2000).

Table1 Important prognostic markers of SCC

Molecular markers	Human*	Canine
TP53/ p53	●	●
MKI67/ Ki67	●	●
Cadherin-1/ CDH1	●	●
Nuclear survinin	●	●
MMPs	●	●
P63	●	
CCND1/ cyclin D1	●	
c-erbB-2	●	
COX-2	●	
VEGF		●
CK	●	●

* Previously reported for SCC prognosis in Non-melanonic skin cancer (NMSC) group (Assawawongkasem et al., 2016; Bardagi et al., 2012; Bongiovanni et al., 2009; Gama et al., 2003; João et al., 2011; Keller et al., 2007; Maiolino et al., 2000; Murakami et al., 2000; Nakaichi et al., 2007; Yan et al., 2011)

2.2.2 Involucrin: candidate marker

Construction

Stratified squamous epithelial cells undergo an orderly process of terminal differentiation that is characterized by specific molecular and

morphological changes, including expression of the cornified envelope protein involucrin (INV). INV is the major soluble small cornified envelope precursors protein (Latin for envelope: involucrum), which was discovered over 35 year ago (Crish et al., 2002; Eckert et al., 1997; Rice and Green, 1977) and had been widely used as a marker of early stage of human terminal keratinocyte differentiation because it specifically expressed only in enlarging cells undergoing irreversible differentiated keratinocyte in suprabasal layer (Balasubramanian and Eckert, 2004; Eckert et al., 1997; Li et al., 2000). Placental mammals have same ancient structure of INV which are homologous segment of 10 amino acid sequences repeats; each repeat contains 3 glutamine residues and segment repeats various by species specific (Tseng and Green, 1990; Vanhoutteghem et al., 2008). Therefore, INV has greatly retains glutamine that make it a good substrate of tranglutaminase for creating insoluble cross-linked envelope beneath the inner surface of the cell membrane by catalyzing heavily bond among INV and other proteins precursor (Eckert et al., 2004; Vanhoutteghem et al., 2008). Moreover, Involucrin after cross-linked are important substrate for ceramide attach and act as an important early scaffold protein of stratified squamous epithelium (Li et al., 2000; Nishifuji and Yoon, 2013; Steinert and Marekov, 1997).

Regulations

Terminal differentiation is characterized by specific molecular and morphological changes which numerous genes are turned on and off at specific stages (Tran and Crowe, 2004). The AP1–binding sequences are promoter/enhancer elements that play an essential role in the induction of many genes in mammalian cells (Eckert et al., 1997; Rorke et al., 2010). Its regulatory factors played a pioneering role in the history of the elucidation of

gene transcriptional regulation and clear that AP1 factors play crucial roles in controlling homeostasis of cell growth including cell proliferation, cell differentiation, cytokine production, apoptosis and oncogenesis (Rorke et al., 2010). Also, two binding sites for AP-1 (activator protein 1) transcription factors (designated AP1-1 and AP1-5) were shown to be important for INV promoter activity and tissue-specific expression. A variety of AP-1 proteins have been shown to interact with and activate the INV promoter, such as c-fos, Fra-1, Fra-2, c-jun, JunB, and JunD (Crish et al., 2006; Takahashi et al., 1998; Tran and Crowe, 2004). Calcium stimulates terminal differentiation of stratified squamous epithelial cells and induces INV promoter activity through interaction with the AP1-5 site. Sp1 site adjacent to the AP1-5 element enhances its activation. However, direct interaction between these transcription factors has not been demonstrated on the INV promoter (Bikle et al., 2002; Eckert et al., 2004). Retinoic acid and Arsenic have been shown to suppress INV expression and promoter activity via the AP1-1 site (Sinitsyna et al., 2010). On the other hand, vitamin A, calcium, and antioxidants have been shown to stimulate INV expression and promoter activity via the AP1-1 site (Eckert et al., 2004). The major upstream signaling pathways of AP1 are MAPKs (mitogen-activated protein kinases) that regulate INV promoter activity and expression. Another one is PKC-dependent that induces INV expression on the distal AP1-5 site (Crish et al., 2006; Eckert et al., 2004; Eckert et al., 1997; Tran and Crowe, 2004; Vanhoutteghem et al., 2008).

Expression

Defective terminal differentiation of transformed stratified squamous epithelial cells has been recognized by decreased INV expression with largely unknown mechanism. However, INV had been studied more in human

dermatology and medical research about normal epidermal renewal structures, patterns and functions, including various immune disease and genetic defect of skin lesion (Bernard et al., 1986; Hertle et al., 1992; Jensen et al., 2004). INV has been known as tumor marker of human SCC as diagnosis and prognosis purpose of premalignant lesion of keratinocytes (Alam et al., 2011; Caldwell et al., 1997; Kanitakis et al., 1986; Li et al., 2000; Murphy et al., 1984; Watanabe et al., 1995). Current model of human tumor has proved that INV are oxygen-regulated protein and useful endogenous marker hypoxia (Chou et al., 2004) and significantly correlated with clinicopathologic sign of SCC patients (Nozoe et al., 2006).

Previously, Tseng and Green (1990) demonstrated INV expression in human, dog and pig in terminally differentiating keratinocyte and explained that are similarly repeat structure of amino acid, but differs significantly with codon sequence of Involucrin gene (Tseng and Green, 1990). However, Kozaki et al. (2001) demonstrated that no positive reaction of INV immunohistochemical stain in dog epidermis, hair follicles and sweat gland duct and did not suggest the use of human INV commercial monoclonal antibodies for investigation dog epithelial tumors (Kozaki et al., 2001). Recently, Theerawatanasirikul et al. (2012) demonstrated positive reaction of INV immunohistochemical staining by using mouse INV commercial monoclonal antibodies, this study showed that among various anatomic sites, breeds and between sexes of normal dog skin were similar staining patterns in INV staining (Theerawatanasirikul et al., 2012b). Moreover, INV mRNA expression was increased significantly in canine atopic dermatitis patients (Theerawatanasirikul et al., 2012b).

Our previous studies demonstrated that even though INV staining in almost cutaneous neoplasm in dogs decreased significantly compared to

normal skin (*p<0.001*), but only SCC groups were significant inverse correlation of INV with proliferative index, Ki67 and histology grading (*p<0.0001*) (Assawawongkasem et al., 2016). In addition, the decreasing levels of INV detected by IHC staining were also related with their mRNA in formalin – fixed paraffin embedded tissues (Assawawongkasem et al., 2016).

2.3 Three dimensional skin and squamous cell carcinoma culturing

Keratinocyte culture techniques have been widely used in dermatological medicine and scientific applications. This is now technically possible for using an *in vitro* method of organic culture of epithelium and keratinocyte to replacement wound and destroy tissue on clinic, called autograft. In research fields, keratinocyte culture model, which is monolayer cultures of one cell type to co-cultures of epithelial and mesenchymal cells and finally to form three dimensional skin equivalents-organotypic cultures, have been developed and needed in experimental researches, including pharmacological test, cosmetic industry, gene therapeutic approaches, and cancer model instead of using animal models, such as use for local invasion and metastasis characterized the biologic behavior of cancers (Inoue et al., 2001; Kuhn et al., 2011).

Keratinocytes can be cultured either from commercial available continues cell line or from donor tissue isolation, as a primary explant. Basically, keratinocyte is a differentiated cells with limited ability to proliferate. Therefore, special conditions should be used to promote their attachment and preserve their differentiate status. Usually it is the proliferating committed precursor compartment of a tissue, such as fibroblast of the dermis or the basal epithelial layer of the epidermis, primary culture especially. Moreover,

keratinocyte culturing should be based on serum-free with low calcium culture media and do not require feeder cells because some growth factors in serum such as transforming growth factor ß (TGF-ß) inhibited epithelial proliferation and favored differentiation. The culturing cells in serum-free permitted the clonal growth of keratinocyte and rapidly remove contamination of other cells such as fibroblasts, and low level of calcium benefit to avoids inducing keratinocyte differentiation. Epidermal growth factor and cholera toxin also should be used to positive effect to keratinocyte proliferation. Keratinocyte require more care, reaching confluence in low density, and slow grown than other cell types. The additional techniques have been concerned to help cells stronger such as for correct matrix interaction, homotypic and heterotypic cell interaction, and the correct cellular polarity (Aasen and Izpisua Belmonte, 2010; Freshney, 2006; Parkinson, 2002). The previous experiments showed that air exposure is an important factor in the proliferation and differentiation of keratinocytes, which is a reason to develop skin culture by air-liquid interface method (Inoue et al., 2001).

Cancer cell culturing can be also established from continue cell line and primary cell line as normal cell culturing with advantage and disadvantage. Even the continuous cell line which is stable to grow, easy to maintain, and commercially available, but it is limited in to some tissue types, species and less mimic to physiological state of cell in vivo and generate less relevant data representing living system than the primary cell culture. Therefore, primary cell culture is commonly used in vitro for pre-clinical and biology mechanism study of epithelial particular cancer (Freshney, 2006; Langdon, 2004). Co-culturing of epithelial cancer cells is useful for local invasion and metastasis for characterizing the biologic cancer behavior. Moreover, cancer cell-stromal cell or cancer cell-extracellular matrix interactions were confirmed that play a

crucial role in malignant growth of cancer cells. Fibroblast is a stromal cell that promote to invasive growth of various cancer cell type, also in local invasion of squamous cell carcinoma into the neighboring tissues (Freshney, 2006; Iwazawa et al., 1996; Kawahara et al., 1993). Inoue et al. (2001) was demonstrated that mesenchymal stromal cell type of dermal fibroblast and subcutaneous adipocytes affect to reduce proliferation and induce differentiation of SCC cell line of the human skin, most probably mediated by adipocytokine. In addition, the authors suggested that air exposure is an appropriate condition to cutaneous squamous cell carcinoma culturing (Inoue et al., 2001).

2.4 RNA interference (RNAi)

RNAi is a common denominator for several post-transcriptional gene silencing (PTGS) processes observed in many eukaryotes, including animals. It plays an important role in defense against parasitic nucleotide sequences – viruses and transposons. The process of RNAi can be moderated by double-stranded RNA (dsRNA), small interfering RNA or short interfering RNA or silencing RNA (siRNA), which considered exogenous double-stranded RNA that is taken up by cells, or enters via vectors like viruses, and single-stranded micro-RNAs (miRNA), which are endogenously expressed nuclear transcripts and processed into shorter hairpin structures, then entering the RNAi process. Both are processed inside the cell by the enzyme called Dicer and incorporated into a complex called RNA-induced silencing complex (RISC). In animals, siRNA binds perfectly to its mRNA target, a perfect match to the sequence, whereas miRNA can inhibit translation of many different mRNA sequences because its pairing is imperfect centered miRNA binding sites. In plants, miRNA tends to have a

more perfect complimentary sequence which induces mRNA cleavage as opposed to just repression of translation. Both siRNA and miRNA can play a role in epigenetics through a process called RNA-induced transcriptional silencing (RITS) (Mack, 2007; Storvold et al., 2006).

siRNA is a class of short double-stranded RNA of 21–23 nucleotides containing 2–3 nt overhangs, 5' phosphates, and free 3' hydroxyl-termini guide the RNAi process resulting in a base-pairing-dependent down regulation of gene expression. siRNA work together with other molecules to destroy target mRNA after transcription. This technique have been used for identification of various diseases and investigate gene function such as co-suppression, quelling, and transgene-induced silencing, even if those processes are not completely identical (Storvold et al., 2006).

RNAi pathway in siRNA using is initiated by the enzyme Dicer (RNAse III nuclease), which cleaves long double-stranded RNA (dsRNA) molecules into short double stranded fragments of ~20 nucleotide siRNAs. Each siRNA is unwound into two single-stranded (ss) ssRNAs, the respective passenger strand and the guide strand. The passenger strand is degraded and the guide strand is incorporated into the RISC. The well-studied outcome is post-transcriptional gene silencing, which occurs when the guide strand pairs with a complementary sequence in a messenger RNA molecule and induces cleavage by Argonaute, the catalytic component of the RISC complex. In some organisms, this process spreads systemically, despite the initially limited molar concentrations of siRNA (Gregory et al., 2005; Jaronczyk et al., 2005; Macrae et al., 2006; Rand et al., 2005; Storvold et al., 2006).

RNAi is a popular technique in genetic engineering researches, both in cell culture and living organism, because synthetic dsRNA introduced into cells can selectively and robustly induce suppression of specific genes of

interest. RNAi may be used for large-scale screens that systematically shut down each gene in the cell, which can help to identify the components necessary for particular cellular process or cellular event such as cell division. The pathway is also used as a practical tool in biotechnology and medicine. Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as a "knockdown", to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated (Macrae et al., 2006; Meins, 2000). Knockdown process of RNAi destroy specific target by no damage gene structure (Alkharouf et al., 2007).

Presently, RNAi is practically used in various fields, for examples in agriculture, use of hpRNA insertion for inhibit glutelin gene syntheses. Whereas, in medical use to support chronic renal failure patient, who need protein control in their feed (Meins, 2000). Inhibiting of CaMxMt1 gene in coffee plantas is practically technique to create decaffeinated coffee (Ogita et al., 2003). Moreover, this technique also use to eliminate crown gall disease due to Agrobacterium tumefaciens infection by inhibit iaam and ipt gene expression (Dunoyer et al., 2006).

RNAi is a promising considered medical treatment by silencing genes, infectious disease especially. For examples, using siRNA combination therapy (SIRCT) for Human immunodeficiency virus-1 (HIV-1) treated by use multi-region siRNA (Berkhout and Haasnoot, 2006). On oncology researches, Rumpold et al. (2005) were reported efficacy application of siRNA to P-glycoprotein inhibiting which benefit to increasing sensitivity of cancer chemotherapeutic agent in progressive cancer cells (Rumpold et al., 2005). In veterinary oncology, the efficacy of survinin gene silencing was reported for increasing apoptosis in canine melanoma (Moriyama et al., 2010).

RESEARCH OUTLINE

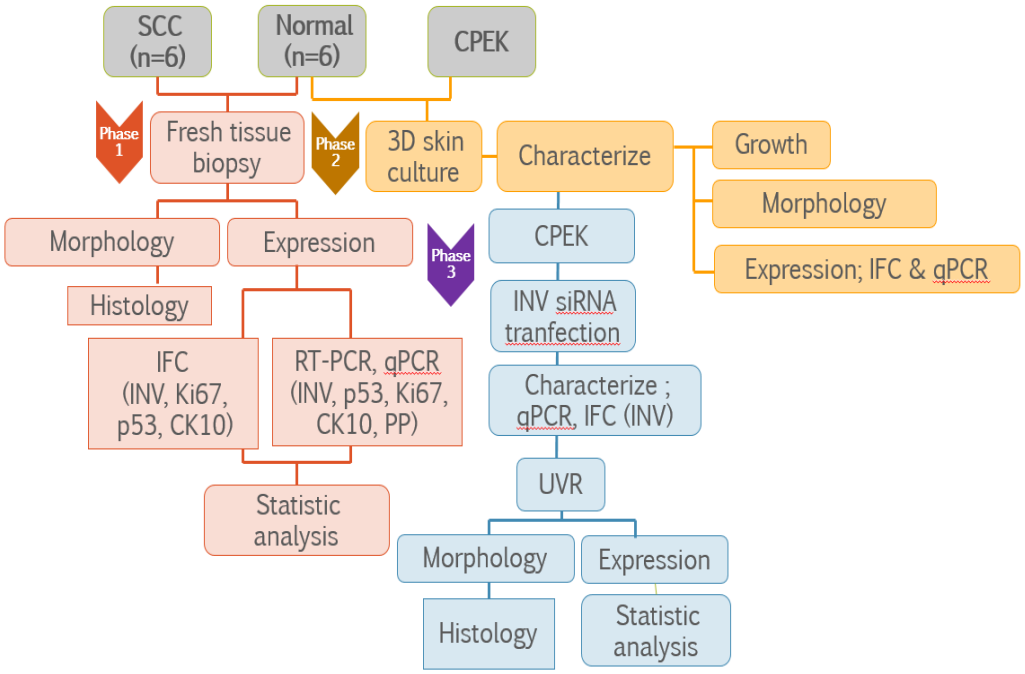

Figure 1 Research outline of this study

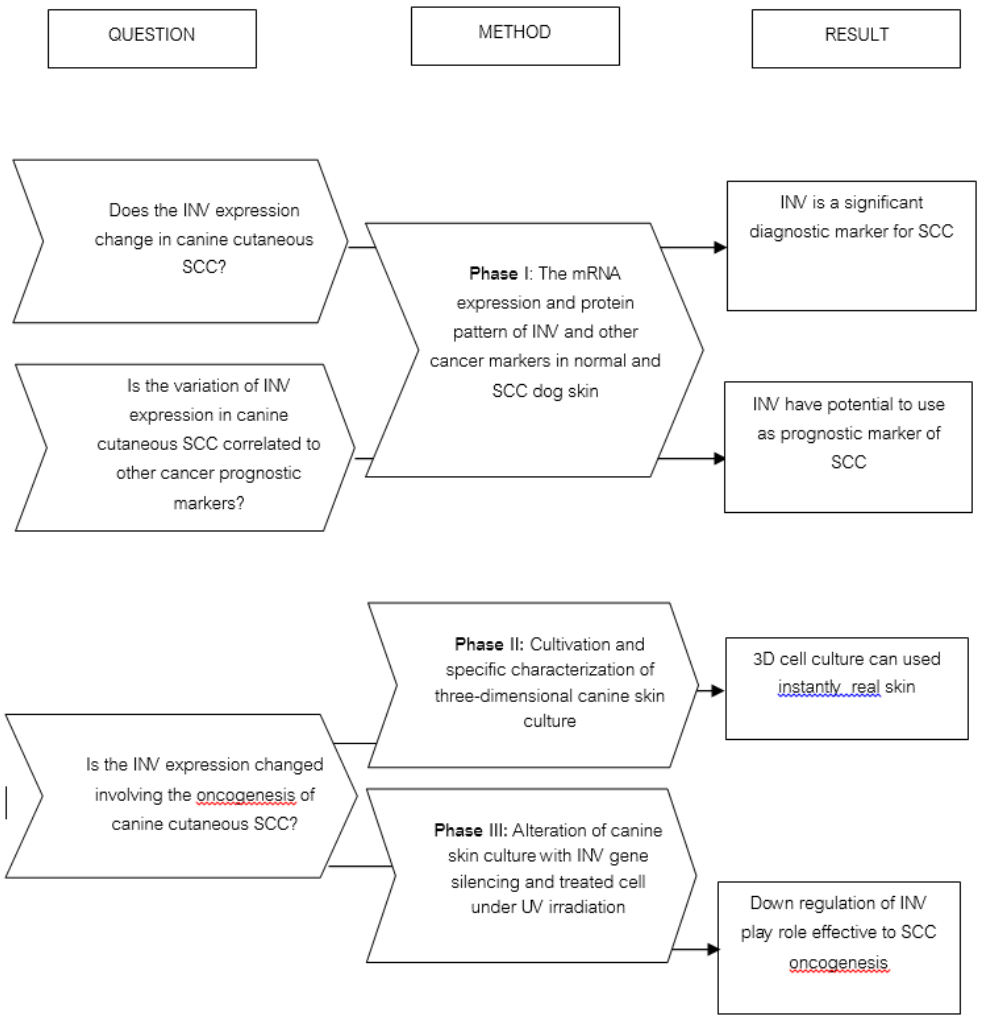

Figure 2 Conceptual framework of this study

CHAPTER III

METHODOLOGY

Phase I: The mRNA expression and protein expression pattern of INV and other cancer markers in normal skin and CSCC in dog

This phase, the determination of INV and other cancer markers expression in fresh normal skin and spontaneous cutaneous SCC in dog were conducted.

1.1 Samples

Six healthy dogs and 6 spontaneous cutaneous SCC (CSCC) patient dogs were obtained by surgical biopsy from the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. The dogs were then operated under the supervision of licensed veterinary surgeons with appropriate anesthesia and under consent of dog owners. The protocol was approved by the Chulalongkorn University Animal Care and Use committee (No.1431049) and the dog's owers agreed to sign the consent form.

The normal skin dogs were collected as control and prior diagnosed used as the samples that they are clinically normal and none of the animal had skin disease involving. Dogs with ectoparasite infestation, skin allergies, bacteria or yeast infection, hormonal imbalances, skin tumors and papilloma virus infection were excluded from this study.

The CSCC patient dogs were then diagnosed by cytology with fine needle aspiration technique for CSCC. The signalment and tumor stages were also collected as below table. All samples were subsequently divided into 4

parts, histology and immunochemistry for INV, immunofluorescence (cryosection), mRNA quantitative and primary cell culture.

Figure 3 Show the gross pathology of the SCC lesions that were collected for the experiment.

1.2 Histology and immunohistochemistry of INV

The skin and CSCC biopsied tissues sized 1x1 cm were fixed in the 10% Neutral buffered formalin (NBF) for routinely histopathology. All formalin fixed paraffin embedded (FFPE) biopsied tissues had been re-cut and routinely processed for hematoxylin-eosin (H&E) light microscopy. Histopathological examination was undertaken according to classification system recommended by WHO (Goldschmidt, 2002).

A serial section of each specimen was prepared for INV-immunohistochemistry using standard protocols previously (Theerawatanasirikul et al., 2012b). Briefly, each deparaffinized tissue section was incubated with citrate buffer (0.01 M, pH 6.0) at 95oC for 40 min for Ki-67 antigen retrieval and trypsinized by 1.0% trypsin for 15 min for INV antigens. The serial sample were incubated with Mouse-monoclonal anti-human involucrin (Clone SY5, Abcam, UK) at the dilution of 1:1000 then incubated by a polymer-based non-avidin-biotin system (EnVisionTM, Dako, Denmark) and

visualized using 3, 3´-diaminobenzidine tetrahydrochloride (DAB) as the chromogenic substrate (Zymed Laboratories, UK). Positive and negative control slides were prepared using canine skin with and without primary antibodies, respectively.

1.3 Immunofluorescence

Routine Cryostat Sections

Collected fresh tissues were immersed in optimum cutting temperature (OCT) compound, Tissue-Tek OCT 4583 compound (Sakura, Tokyo, Japan) and rapidly frozen by dry ice. Then 6- μm sections were cut using a routine cryostat microtome, Leica CM1800 cryostat (Leica, Heidelberg, Germany) at -20oC. Each pieces of cryostat tissue was placed in normal slide and methylene blue stained before for observed best position and thickness.

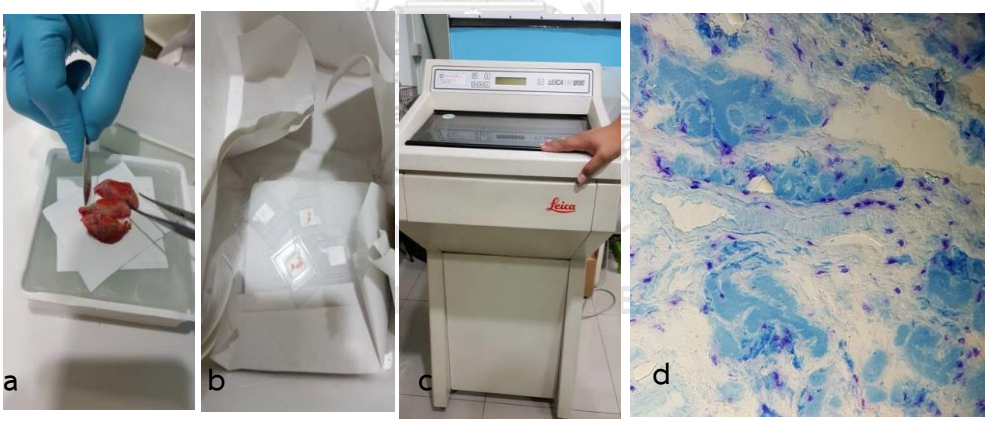

Figure 4 The fresh biopsy was aseptically collected from the dog and placing on the cryosection cassette in dry ice. The OCT was then dropped on the tissue for freezing the section (a,b). All the cutting processes were performed in the cryosection machine at -20oC. The cryostat sections were stained with methylene blue for checkup the section and the thickness before immunofluorescence staining (c,d).

Immunofluorescence microscope

Immunofluorescence were performed on 6- µm cryostat sectioned normal skin and cutaneous SCC on coated slide, as XL Biotech Co, Ltd (Thailand) protocol. The sections were fixed by Fixing I, 15 min in dry ice. Then, on Fixing II 20 min at 0 °C, and then incubated with staining blocking (FC blocking buffer, ratio4:100) 100ul/slide at 4°C for 45 min, and then incubated with 100 µl/slide of primary antibodies mouse/rabbit monoclonal antibody against INV (SY5, Abcam), Ki67 (MIB1, Santa Cruz biotechnology), cytokeratin (CK) 10 (RKSE60, Abcam), and p53 (Pab 246, Santa Cruz biotechnology), mouse IgG1. Mouse IgG2 were used as isotype control at 4oC for 60 min, and then washed with antibody binding buffer 5 min, 3 times. The tissue were incubated with secondary antibodies (Abcam), rat anti-mouse/rabbit FITC, Texas red, and Cy5 conjugate were used at 4oC for 60 min, and then washed with antibody binding buffer 5 min, 3 time. DAPI were used for nucleus counterstaining at the same time. The immunolabeled sections were mounted with glycerol/PBS and observed under confocal LSM (Leica DFC7000T, Germany), wavelength of 488 nm (for FITC), 543 nm (for Texas Red), and 649 nm (for Cy5) were used as the excitation source. Color images were acquired as optical sections and processed with LAS X imaging software which is part of the equipment of fluorescence microscope.

1.4 mRNA quantitation

The 1x1 cm of skin tissue samples in RNA later solution were extracted using RNAzol®RT (Sigma®) according to the manufacturer's instructions. Total RNA solution of each specimen were subsequently treated to diminish genomic DNA from the RNA solution by TURBO DNase-free (Ambion, USA) and quantified by the Nano Drop ND-1000 Spectrophotometer

V3.7 (Thermo FisherScientific, USA) to determine the concentration and purity of the RNA samples by A260. Then 10 ng of total RNA were treated with DNase Free reagent (Ambion, Austin, TX) for 60 min and reverse-transcribed with SuperScript®III platinum® SYBR® Green one step qRT-PCR kit (Invitrogen, USA) at 37 ℃ for 120 min using random primers. Using 5 µl of the reverse transcription reaction as a template, primer set for canine genes (Involucrin, CK10, p53) were designed using Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/) following a previous study (Theerawatanasirikul et al., 2012a). The papilloma virus in samples were investigated by using two primer sets. These primers were MY09/MY11 primers (PP1) and FAP59/FAP64 primers (PP2) and amplifications as previously described (Paolini et al., 2013; Waropastrakul et al., 2012), with human oral papilloma in FFPE used as control. All primers information show as table 2. Quantitative real time PCR were performed using Swift® spectrum 48 Real Time Thermal Cyclers (Esco, Singapore) with Taqman reagents (Applied Biosystems, Foster City, CA). ß-actin as housekeeping genes (Kappa) were used as internal controls to normalize expression of genes of interest by the **ΔΔ**Ct method (Wong and Medrano, 2005). Standard curves were generated using the data for 10 fold serial dilutions of total RNA to confirm efficiency of the assay, and great R^2 value.

Table 2 The primers for qPCR in this study

Gene	Primers	Product (bp)	Reference
INV	Fwd 5'AAA GAA GAG CAG GTG CTG GA 3' Rev 5'TGC TCA CTG GTG TTC TGG AG 3'	203	*Theerawatanasirikul et al., 2012*
CK10	Fwd 5'TTG AGA CGC ACT GTT CAA GG 3' Rev 5'AGC TCG GAT CTG TTG CAG TT 3'	168	*Theerawatanasirikul et al., 2012*
Ki67	Fwd 5'-CCCACCTGTCCTGAAGAAAA-3' Rev 5'-TGTGGTCACTTCCAGTTGGTT-3'	88	*Vascellari et al., 2012*
p53	Fwd 5-CTGGCTAGACGAAGACTCAG-3 Rev 5-AGGCAGTGCTCGCTTGGTAC-3	738	*Koenig et al., 2002*
PP1	Fwd GCMCAGGGWCATAAYAATGG Rev CGTCCMARRGGAWACTGATC	452	*Waropastrakul et al., 2012*
PP2	Fwd 5'-TAACWGTIGGICAYCCWTATT-3' Rev 5'-CCWATATCWVHCATITCICCATC-3'	484	*Waropastrakul et al., 2012*

(Koenig et al., 2002; Theerawatanasirikul et al., 2012a; Vascellari et al., 2012; Waropastrakul et al., 2012)

1.5 Statistic analysis

Statistical analysis of immunofluorescence were performed by using image analyzer of laser confocal microscopy. Differences in distributions of INV and other markers among groups were analysis using relative quantification. For the matched normal and SCC analysis, we were used One-way analysis of variance (ANOVA) & Turkey-Kramer test. All statistics were calculated by using SPSS version 15 for Windows (IBM Corporation, New York, USA). Mean, standard deviation, and coefficient of variation of each marker were determined. $P<0.05$ were considered statistically significant.

Phase II: Cultivation and specific characterization of three-dimensional canine skin culture

This phase, the development of cultured of primary normal skin, spontaneous SCC, and 3-dimensional cultured model of commercial canine keratinocyte cell line for assessing INV and other markers of protein antigens and RNA expressions were conducted.

2.1 The culture of normal skin, spontaneous cutaneous SCC and establishment of three-dimensional (3D) skin culture

The cultured cells were divided into 3 groups which were isolated from samples of group 1: 3 normal canine skins (CK), group 2: 3 spontaneous CSCC using biopsies and diagnosed without papilloma virus infection, and the other was a commercial canine keratinocyte cell line (CPEK) (CELLnTEC Advanced Cell Systems, Switzerland). The primary cell culture group1 and 2 (CK, CSCC) were performed by primary explant technique (Orazizadeh et al., 2015), the biopsy samples were kept in transfer medium during transportation and immediately started to cell culturing. The samples were then washed 3 times with PBS. The samples were cut into 2-3 mm pieces and digested in 0.25% trypsin at 4 °C overnight. After epidermal layer was separated from the dermis, explant pieces were placed in CnT-09 medium (CELLnTEC Advanced Cell Systems, Switzerland).

Day 1, medium was added to each Petri dish and day 4 the explants were removed and medium was then added. Subsequently, the cells in the Petri dish were maintained in this condition until the 6th day. On the 6th day, the whole medium was removed and replaced by fresh medium. Cells were checked and medium changed daily until harvested. The cells were incubated at 37 °C in 5% CO2 atmosphere.

For group 3 of CPEK were maintained in complete CnT-09 medium (CELLnTEC Advanced Cell Systems, Switzerland). The 3D culture was performed on the basis of protocol reported by Yagihara et al. (2011) (Yagihara et al., 2011). The cell were thawed and harvested newly passage to cultivating The CnT-09 medium were used and changed every 2 day without antibiotic/antimycotics at 37 °C in 5% CO2 atmosphere. Day 3-4, the cell were reached to 80% confluence. The cells were washed and transferred to Millipore PCF polycarbonate insert (0.4 μm pore size, 12 mm in diameter) with seeded of viable cells at 5x105 cell/ml. Inserts were placed in multi-well plate and immersed with CnT-PCT medium (3D culture medium) till the medium level inside and outside the insert equally and submerged the cells. This technique is called as air-liquid interface. The cells were cultured until Day 21 with the medium changed alternate day. Culture specimens were collected on Days 14 and Day 21.

2.2 Characterizations of the cultured cells

Growth rate and Morphology

Growth rates of monolayer cell culture of keratinocyte were observed daily before performing 3D culture. The cells were harvested for cell counting by a hemocytometer under phase contrast microscopy. Viability was evaluated by the trypan blue exclusion method or Fluorescence-base Molecular ProbesTM LIVE/DEADTM assay (ThermoFisher®) were used (double staining contains calcein-AM and propidium iodide (PI) solutions, which stain the viable and dead cells, respectively). After 3D culture, the cultured samples were fixed in 10% NBF and embedded in paraffin at days 14 and 21 in culture for routine histology technique.

Histological observation was processed with hematoxylin and eosin (H&E) for estimating growth of cell 3D culture under light microscopy.

Immunofluorescence microscope

The method followed the immunofluorescence as in 1.3 and the samples were stained with INV, Ki67, CK10, p53, mouse IgG1. The mouse IgG2 was used as isotype control.

mRNA quantitation

The samples in inserts were extracted using RNAzol®RT (Sigma®) according to the manufacturer's instructions. Total RNA solution of each specimen were subsequently treated to diminish genomic DNA from the RNA solution by TURBO DNase-free (Ambion®, USA) and quantified by the Nano Drop ND-1000 Spectrophotometer V3.7 (ThermoFisher Scientific, USA) to determine the concentration and purity of the RNA samples by A260. Then 10 ng of total RNA were treated with DNase Free reagent (Ambion, Austin, TX) for 60 min and reverse-transcribed with SuperScript®III platinum® SYBR® Green one step qRT-PCR kit (Invitrogen®, USA) at 37 °C for 120 min using random primers. Using 5 µl of the reverse transcription reaction as a template, primer set for canine genes (Involucrin, CK10, p53) were designed using Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/) following a previous study (Theerawatanasirikul et al., 2012a). Quantitative real time PCR were performed using Swift® spectrum 48 Real Time Thermal Cyclers (Esco, Singapore) with Taqman reagents (Applied Biosystems, Foster City, CA). The ß-actin as housekeeping genes (Kappa®) were used as internal controls to normalize expression of genes of interest by the **ΔΔ**Ct method (Wong and Medrano, 2005). Standard curves were generated

using the data for 10 fold serial dilutions of total RNA to confirm efficiency of the assay, and great R^2 value.

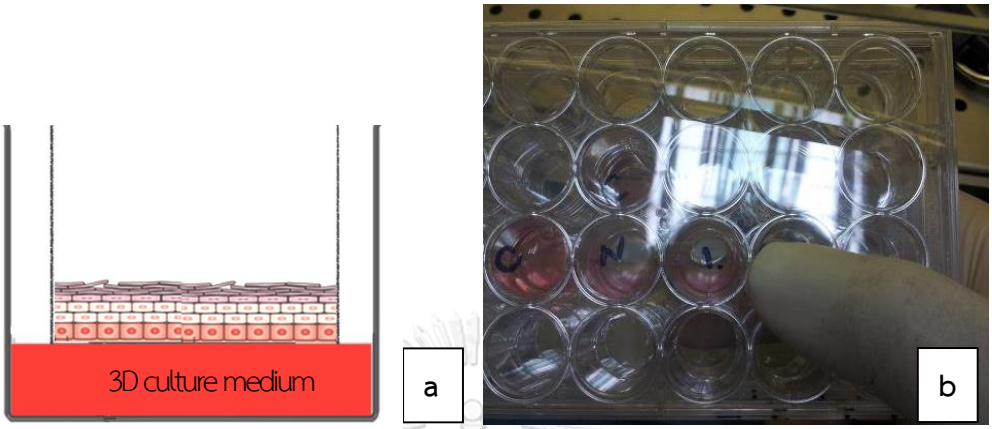

Figure 5 The air-liquid interface cultured technic. a) diagram of culturing system b) The Millipore PCF polycarbonate insert at 4µm pore size and 12 mm diameter with seeded of the CPEK viable cell at 5x105 cell/ml were placed in the microwell plate with CnT-PCT medium or 3D culture medium. The medium level inside and outside the insert are equally and submerging the cells.

2.3 Statistical analysis

On considering culture modeling reliability, the result of each protein expression staining pattern per area in fresh tissue section (Phase I) was compared with those expressions in the developed culture. The results of protein expression from image analyzer of laser confocal microscopy were calculated by using SPSS version 15 for Windows (IBM Corporation, New York, USA). The independent t-test (significance set at P 0.05) was used to test the difference between each group of cryostat section and cell culture.

For the CSCC- CK analysis, we were one-way analysis of variance (ANOVA) & Turkey-Kramer test used.

Phase III: Alteration of canine keratinocyte culture with INV gene silencing and treated cell under UV irradiation

This phase aimed to prove the hypothesis that whether INV is an important parameter on oncogenesis of cancer. The INV siRNA-treated cell on CPEK was prepared and compared the pathological changing of treated cell with normal CPEK under prolonged UVB irradiation induced apoptosis. This results were indicated the possibility on using of INV effect on the oncogenesis of cutaneous. This model was conducted in CPEK cell.

3.1 siRNA design and titration

Double-stranded, siRNAs (21-mer) targeting INV were designed and synthesized by web-based program of Bioneer's Custom siRNA synthesis service (https://us.bioneer.com/sirna/custom-sirna-ex.aspx). Targeted UTR regions of INV were designed and top 3 candidates of INV siRNA designed were selected and synthesized as INV-1, INV-2 and INV-3 as shown in table 3.

Table 3 Target mRNA sequence of designed siRNA of involucrin in dog

INV-1 (20 nmol)	
SENSE	GAA CAG CUG GAA CAG GAA A (dTdT)
INTISENCE	U UUC CUG UUC CAG CUG UUC (dTdT)
INV-2 (20 nmol)	
SENSE	GCU AGA GCA GAU AGG AGC A(dTdT)
INTISENCE	U GCCU CCU AUC UGC UCU AGC(dTdT)
INV-3 (20 nmol)	
SENSE	AGC ACC AGA AGC AGG AGG U(dTdT)
INTISENCE	A CCU CCU GCU UCU GGU GCU(dTdT)

For the siRNA treated seed condition, cultures were transfected with a transfection reagent using a proprietary dosing strategy with a siRNA concentration ranging from 50, 100, 150, 300 and 600 picomoles/106 cells for INV-1, INV-2, INV-3 and pool 3 types of siRNA. The appropriated condition of siRNA transfection or titration were investigated with day 3 of seed cultures in monolayer culture of CPEK (at 40-60% cell confluence) followed as description of AccuTarget® Custom siRNA guidline (www.bioneer.com).

Transfection of siRNA were performed using lipofectamin™ RNAiMAX (Invitrogen®, USA) according to the manufacturer's protocol. At approximately 40-60% confluence of monolayer culture of keratinocyte in group C-D were transfected with either INV or control siRNA using appropriate reagent according to the manufacturing's instructions and preliminary trials. After 5 h of transfection, the transfection mixed were washed out and medium added again, and then were started harvesting in 0, 6, 12, 24, 48 h after added medium to observe their growth rate and characteristic under phase contrast microscopy. All of cell groups were divided in to 2 groups; to RNA isolation for detecting change of INV after siRNA treated CPEK and compare with control group and cell vitality staining by LIVE/DEAD® Viability/Cytotoxicity Kit (ThermoFisher Scientific). Negative control was non-transfected cell. Triplicates samples were conducted in each group.

After obtained appropriate concentration and time, the condition was selected to seed transfected keratinocyte onto 3D cultures as followed in 2.1. Briefly, The cell in medium was changed every 2 day until day 3-4, the cells were reach to 80% confluence, then washed and transferred to millipore PCF polycarbonate insert with seed viable cells at $5x10^5$cell/ml. Inserts were placed in multi-well plate and immersed with 3D culture medium. The cells were cultured until Day 14 with the medium changed alternate day.

3.2 UVB-induce apoptosis model

After the preparing siRNA treated CPEK, was developed the cell from A-D groups to UVB-induced apoptosis were performed. The normal three dimensional (3D) CPEK cell culture were prepared, seeded in 12 well plates for 4 plates and divided into 4 groups for preparing experimental as below;

Group A: CPEK –control (control)

Group B: CPEK –UVB (Control+UV)

Group C: siRNA-treated CPEK (si)

Group D: siRNA-treated CPEK –UVB (si+UV)

Cells in plate of group B and D were UVB irradiated at a dose of 300 mJ/cm^2 UVB with a DNA crosslinker light (Spectrolinker^{TM} XL-1000 UV crosslinker, USA) following method of previous study (Keller et al., 2007). The cells with an emission peak at 312 nm. Each group were irradiated at different time points. The subsequent irradiations followed at 1-h intervals, the final dose was delivered after 24 h. During irradiation, the culture medium was replaced with phosphate-buffered saline (PBS); thereafter the cells were further incubated in culture medium. All cultures were harvested simultaneously at 24 h after experiment and were further processed together for characterizing of morphology, fluorescein staining and expression of INV, p53, CK10 and Ki67 mRNA. Cells in plate of group A, C were not exposed to UV irradiation served as negative controls of each group.

Figure 6 The UV irradiation machine (Spectrolinker™ XL-1000 UV crosslinker, USA), with UVB light at 312 nm. The exposure was performed in the laminar flow.

3.4 Statistical analysis

All statistical analyses were performed using SPSS version 15 for Windows (IBM Corporation, New York, USA). Mean, standard deviation, and coefficient of variation were determined. Comparing of Involucrin level between siRNA transfected CPEK and control CPEK, and Comparing among group A-D were assessed by using independent t-test (significance set at $P<0.05$).

CHAPTER IV

RESULTS

Phase I: Differentiation of mRNA expression and protein pattern of Involucrin and other cancer markers in normal and SCC dog skin

1.1 Signalments of normal and CSCC dogs

The samples in this study were collecting from 6 healthy dogs (1 males, 5 females) and 6 spontaneous cutaneous SCC (CSCC) patient dogs (3 males, 3 females) which were obtained by surgical biopsy from the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. The normal dog's information (n=6) showed as tables 4 and the CSCC patients dog's information (n=6) showed as table 5. The CSCC samples were collected from the dog age range at 5-15 year and the clinically normal dog, healthy, no skin problem from early birth to 8 year of age.

Table 4 The clinically normal dog's information (n=6)

Breed	Age (Years)	sex	Collection site
Shih Tzu	early birth	female	body
Mixed	1	female	abdomen
Mixed	8	female	abdomen
Pomeranian	5	male	back
Chihuahua	1	female	abdomen
Shih Tzu	7	female	back

Table 5 The CSCC patients dog's information (n=6)

Breed	Age (Yrs)	Sex	Site	Duration of CSCC growth	SCC grading *(Goldschmidt MH and Hendrick, 2002)*
Shih Tzu	8	male	Front leg	10 month	Well- differentiated
Mixed	12	female	Hind limb digit	3 month	Poor- differentiated
Mixed	5	female	Front leg	No observed	well- differentiated
Schnauzer	9	male	back	1 year	well- differentiated
Golden retriever	8	male	Front leg	1 year	moderate- differentiated
Poodle	15	female	Ventral	3 month	well- differentiated

1.2 Histology, immunohistochemistry and immunofluorescence characterizations

1.2.1 Normal canine skin

Histological appearance of normal skin consisted epidermis, and dermis. The epidermis composed of several layers of epithelium, including keratin layer in upper layer with eosinophillic color and lower is a granular layer, spinous layer and basal layer, respectively. The dermis presents below the epidermal layer, contains smooth muscle, blood and lymphatic vessels and variably sized of collagen and elastic fibers (figure 7a).

Immunohistochemistry (IHC) staining pattern of INV demonstrated a strong granular like intra-cytoplasmic pattern in the whole layer of the nucleated epidermis in upper spinous and granular layer of the stratified squamous epidermis (figure 7b). Immunofluorescence (IFC) staining pattern using computer color merged program (Microscope software platform LAS X,

Leica) (figure 7c). The cytokeratin 10 (CK10) (figure 7d) and INV (figure 7e) was positive intra-cytoplasmic pattern in whole layer of nucleated epidermis, the epidermis nuclei were stained with DAPI (figure 7f). Intra-nuclei of epidermal cells were positive with Ki67 as the red dot (figure 7g), but negative for p53 (figure 7h) in all samples.

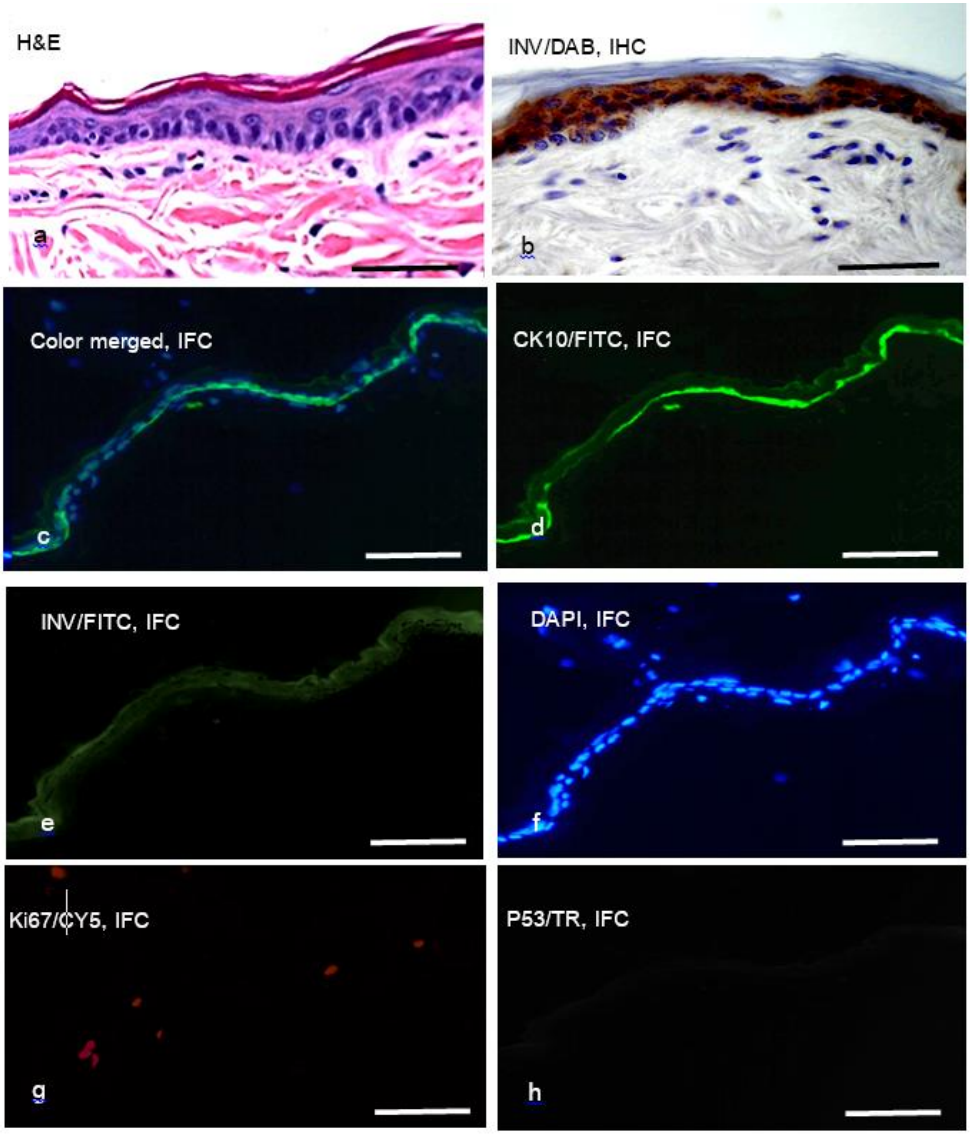

Figure 7 The histology, immunohistochemistry and immunofluorescence staining pattern in canine skin, (a) H&E (b) INV antibody, DAB, hematoxylin counterstaining, IHC (c) Color merged, CK10/Ki67/p53, IFC (d) CK10/FITC, IFC (E) INV/FITC, IFC (f) DAPI, IFC (g) Ki67/cy5, IFC (h) p53/TR, IFC; Color/Bar= 25 µm.

1.2.2 Canine cutaneous SCC (CSCC)

The 6 CSCC of dog patients were undertaken using the classification system recommended by WHO (Goldschmidt, 2002), wihich consisted of 4 well-differentiated SCC, 1 moderate-differentiated SCC and 1 poor-differentiated SCC.

Histolopathology appearance of CSCC consisted of large neoplastic keratinocytes. The nuclei were hyperchromatic and nucleoli varie in size and prominently. Formation of keratin pearls and intracytoplasmic keratin proteins were well identified. The Well-differentiated SCC, proliferating neoplastic squamous epithelial cells, arranged in compact cords or nests of varying in size, abundant connective tissue and lamellate keratin pearls in the center of the islands (figure 8a). Moderate-differentiated SCC, compactly arranged proliferating cells forming cords or nests of cells separated by thin fibrous stroma with presence of individual keratinized cells (figure 8b). Lastly, Poor-differentiated SCC, highly proliferating cells showing high anaplastic with absence of cell nests and keratin pearls or keratinized cells (figure 8c).

Immunohistochemistry (IHC) of INV were revealed low staining pale brownish color at keratin pearl area with DAB-hematoxylin counterstained (figure 9b).

Immunofluorescence (IFC) staining pattern using computer color merged program (Microscope software platform LAS X, Leica) (figure 9c). The cytokeratin 10 (CK10) (figure 9d) were strong positive along intracytoplasm and INV were positive weakly in some area (figure 9e), the nucleus stained with DAPI (figure 9f). Intranuclear staining were strongly, when compared to positive with Ki67 and p53 normal skin.

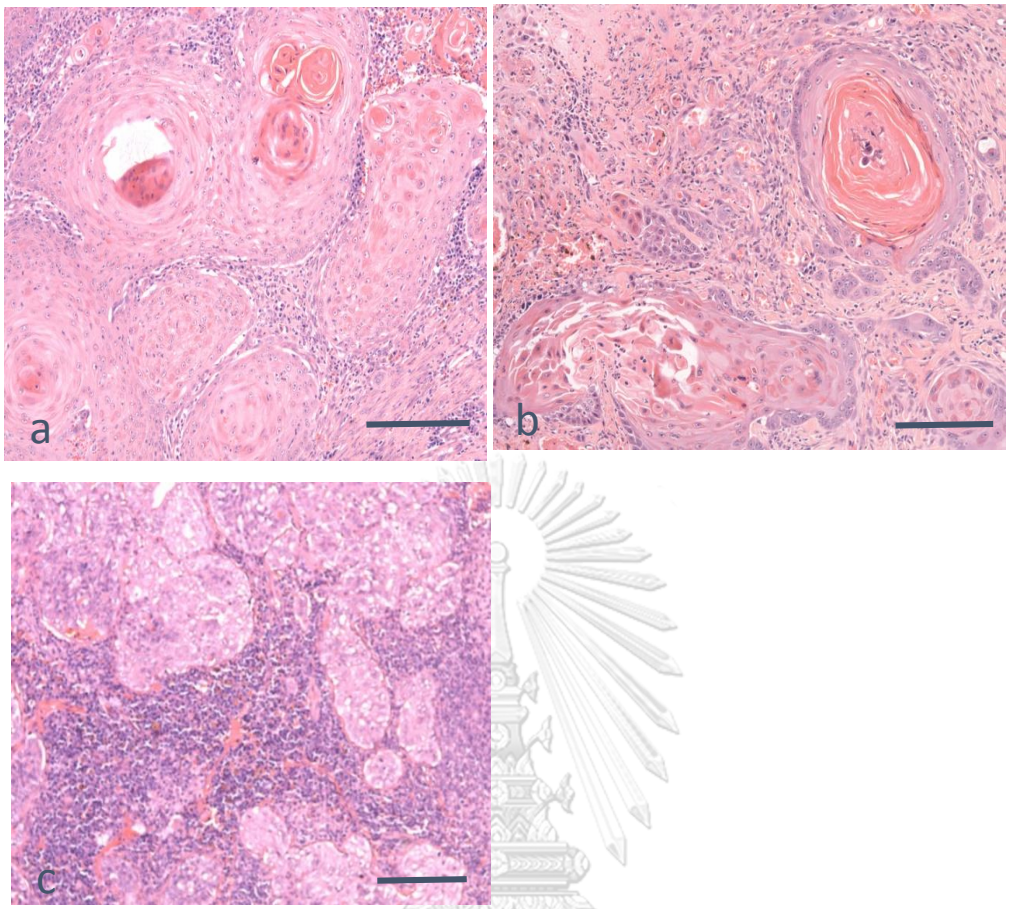

Figure 8 Histology characteristic of CSCC classification system recommended by the WHO (Goldschmidt, 2002) (a) Well-differentiated SCC (b) moderate-differentiated SCC and (c) poor-differentiated SCC. (H&E), Bar=25 μm.

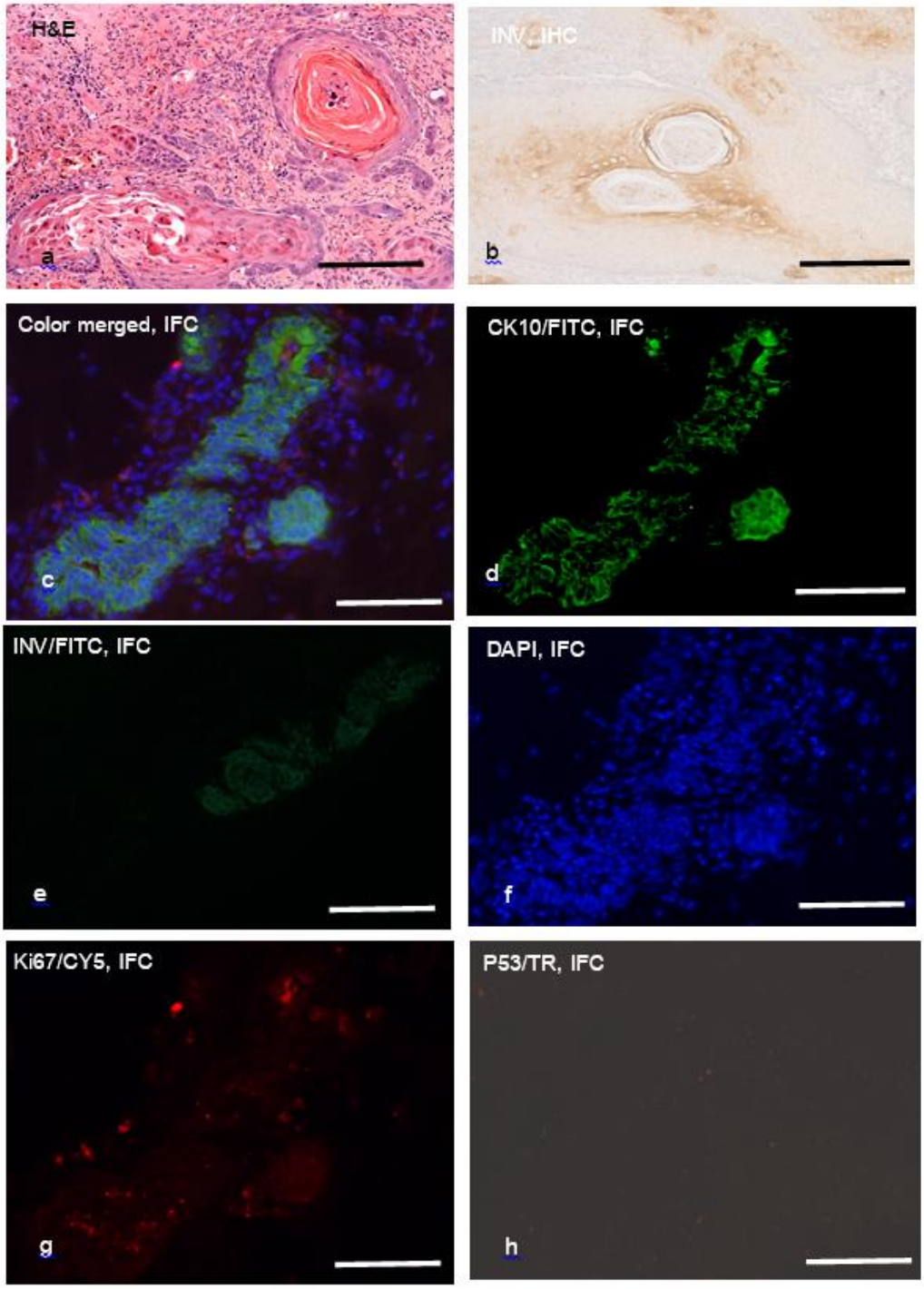

Figure 9 The histology, immunohistochemistry and immunofluorescence staining in spontaneous CSCC (a) H&E (b) INV antibody, DAB and hematoxylin counterstaining, IHC (c) Color merged, CK10/Ki67/p53, IFC (d) CK10/FITC, IFC (e) INV/FITC, IFC (f) DAPI, IFC (g) Ki67/cy5, IFC (h) p53/TR, IFC; Color/Bar= 25 μm.

1.3 mRNA expression

The mRNA quantitation, comparing the scores of the mRNA expression of INV, p53, CK10, Ki67 in the normal skin and CSCC in dog of Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. The mRNA expression by qPCR are in parallel with immunofluorescence staining pattern. The mRNA expression of each parameter in normal skin in comparison to CSCC revealed INV expression of normal skin is more than CSCC while Ki67 in normal skin less than CSCC. The papilloma virus (PP1, PP2 primer) is negative in all samples. Though, p53 has not shown any statistically difference, the trend of mRNA expression of SCC tends to be likely more than normal skin.

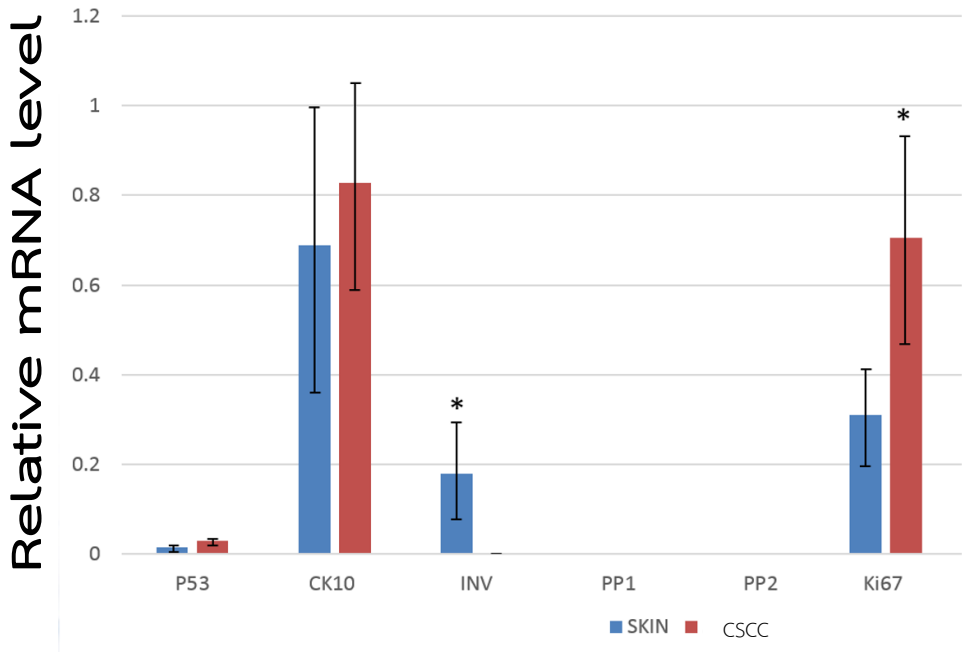

Figure 10 The histogram comparing the scores of the mRNA expression of INV, p53, CK10, Ki67 in normal dog skin and CSCC in dog. Differentiation significant showed that INV in normal skin more than SCC and Ki67 in normal skin less than SCC. Negative of papilloma virus (PP1, PP2 primer) in all sample (n=6 of each) (mean+SD). Symbol * showed statistically significant differences ($p<0.05$). ß-actin as the internal control.

Phase II: Cultivation and specific characterization of three-dimensional keratinocyte skin culture

2.1 Three-dimensional (3D) keratinocyte (CPEK) culture

2.1.1 Histological and immunofluorescence characterizations

The cultured CPEK cell monolayer by using Cnt-09 media was successfully conducted by producing 80-90% confluently appearance (figure 11). However, it was found that by using the same media the primary cell culture both normal skin and CSCC could not obtained good result of keratinocyte. On observation, the cultured CPEK demonstrated confluent appearance at 80% in 3-4 days and starting for 2^{nd} subculture, changed the media every 2 days without antibiotic/antimycotic at 37°C in 5% CO2 atmophere. After 80% confluence cells, the cells were then harvested, subsequently washed and transferred to millipore PCF polycarbonate insert and then were placed in multi-well plate and immersed with CnT-PCT medium (3D culture medium), until the medium level inside and outside the insert equal and then submerged were the cells. This technique is called as air-liquid interface. The cells were cultured for 14 day. At day 14, the cells revealed well proliferation and undergone the differentiation appearance of the epidermal characteristic like the skin (Figure 12a). The culture continued until 21 days. And it was found that the cells ceased and starting to degenerate (Figure 12b). For the immunofluorescence staining, the INV, CK10 visualized the nucleus with DAPI, revealing the positive results while ki67 and p53 showed negative result (Figure 13).

By using the CnT-PCT (CnT-09) media, the primary culture of skin and CSCC could not be successfully performed. In primary cell culture of normal skin in

dog in Cnt-09 using primary explant technique; the primary cell growth appeared a slow growing with some scattering of keratinocyte around the skin. These cells were undergone degeneration, sloughing slowly and dead at Day 30. The fibroblast had still appeared and rapidly proliferated on 4-7 day (Figure 14). In primary cell culture of canine CSCC, The cultured CSCC cells performed under the same technique; the primary cell growth appeared a rapid growth pattern and proliferating on day 3, it did not adhere the cultured dish, became clumping and contamination with bacteria on day 5-7 (Figure 15).

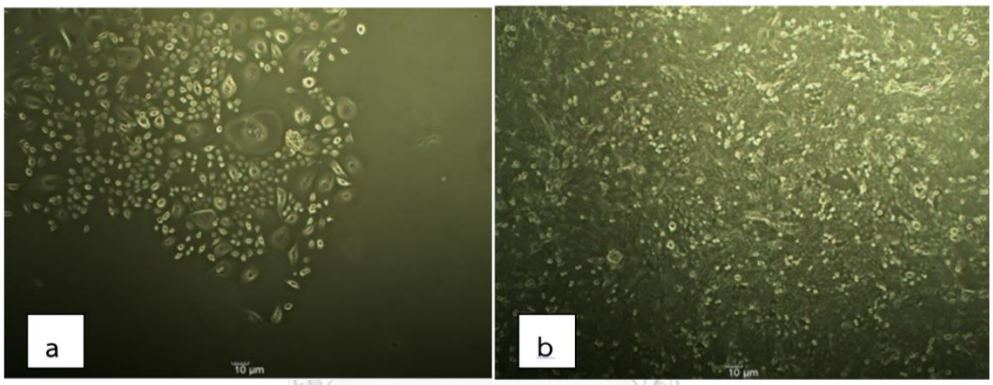

Figure 11 The morphology of CPEK in culture; cell growth in the Cnt-09 media on Day3 (a) and Day 5 (b), the CPEK demonstrated a well growth pattern, there were many cell morphologies as rounded large in the squamous like and short elliptical cells. Phase contrast microscope (Zeiss® microscope) with Moticam 3.0mp digital camera, Bar=10 µm

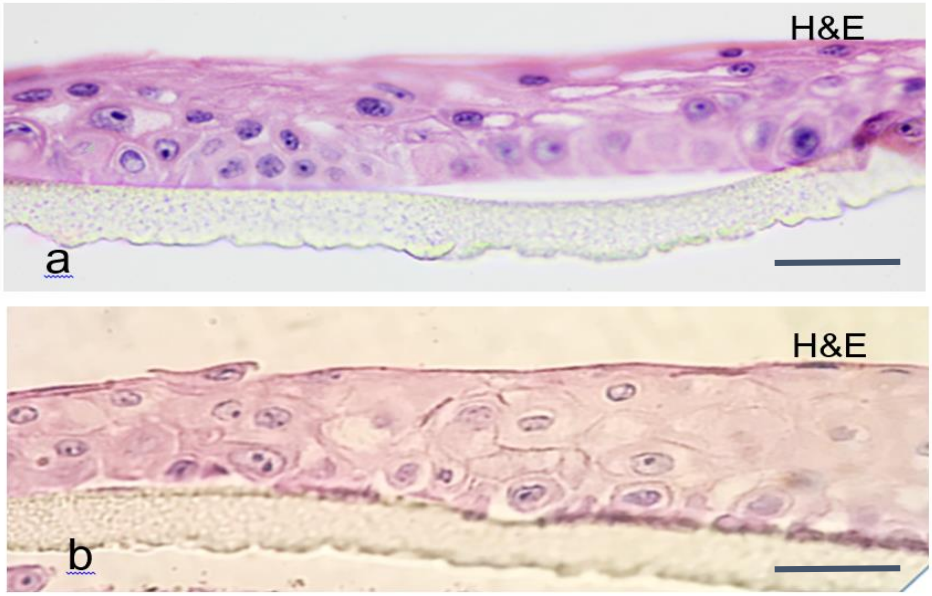

Figure 12 The histopathology of three-dimensional (3D) air liquid interface cultured canine keratinocyte (CPEK); the 3D cultured cells became a stratified squamous epithelium as epidermal like structure on the Millipore PCF polycarbonate, on Day 14 (a) and Day 21 noted that the keratinocyte became swollen and started to degenerate (b). H&E, Bar= 25 μm.

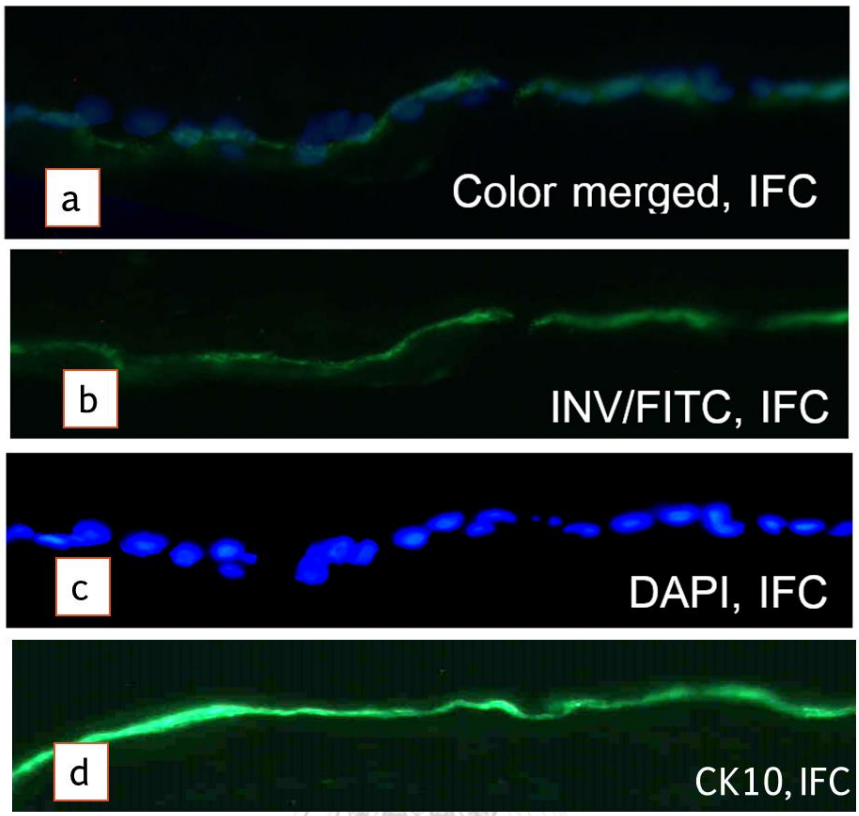

Figure 13 Immunofluorescence staining (IFC) of 3D air-liquid interface cultured canine keratinocyte (CPEK); color merged (a), INV/FITC (b), for nuclear demonstration, DAPI (c), Cytokeratin10/FITC, Leica DFC7000T with LAS X imaging software, IFC (X400)

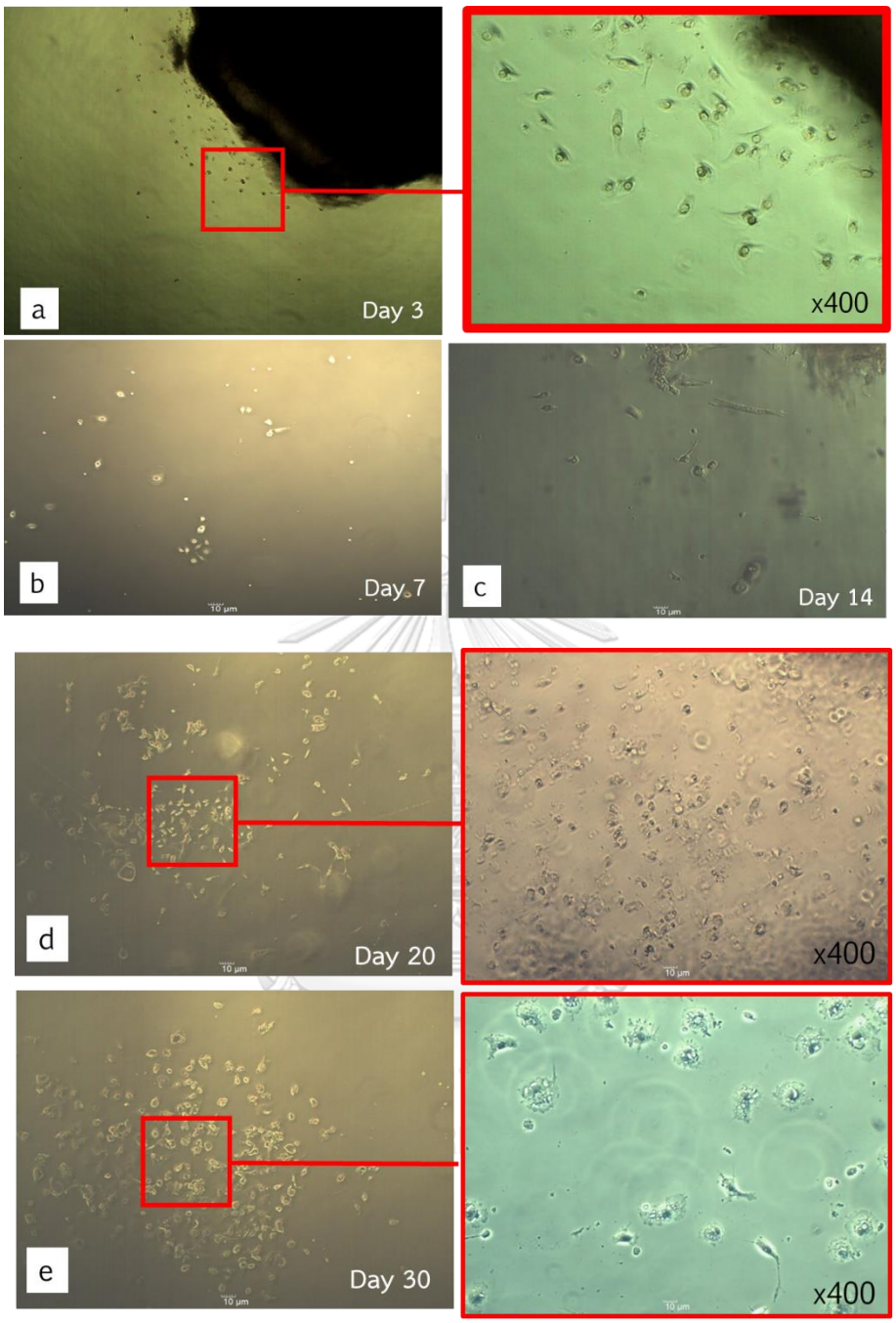

Figure 14 The morphology of primary cell culture of normal canine skin (abdominal site) in CnT-09 using primary explant technique; the primary cell growth appeared a slow growing with some scattering of keratinocytes around the skin (a) and higher magnification (red frame on the right side of figure, HP) these cells were undergone degeneration and sloughing slowly (b,c,d) and dead at Day 30 (e). Noted that fibroblast appeared and rapidly proliferation on 4-7 day, Phase contrast microscope (Zeiss® microscope) with Moticam 3.0mp digital camera, x100.

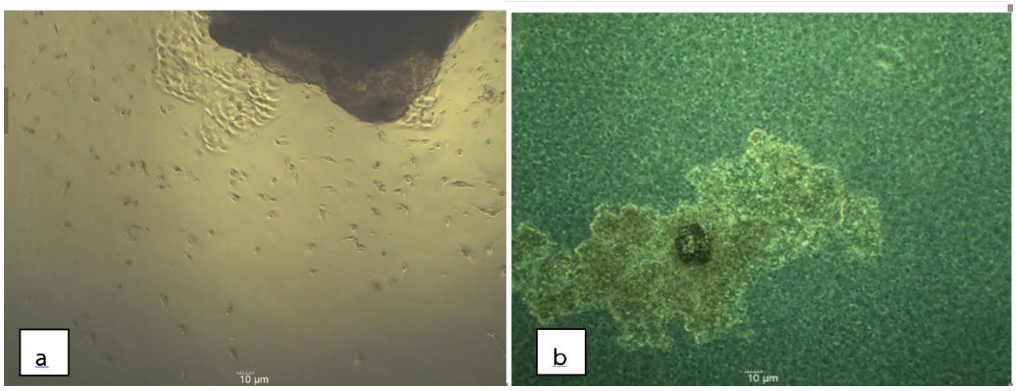

Figure 15 The morphology of primary cell culture of canine cutaneous SCC in Cnt-09 using primary explant technique; the primary cell growth appeared a rapid growth pattern and proliferating on day 3 (a). The cultured CSCC cells did not adhere the cultured dish, became clumping, aggregation and contamination with bacteria on day 5-7 (b). Phase contrast microscope (Zeiss® microscope) with Moticam 3.0mp digital camera, x100.

2.1.2 mRNA expression

The mRNA quantification, when comparing with the scores were agreed to the results of immunofluorescence staining appearance of INV, CK10 were no statistical difference of INV, CK10 between 3D cultured of CPEK. However, upon the experiment the results of p53 and ki67 in CPEK not presented. (Figure 16)

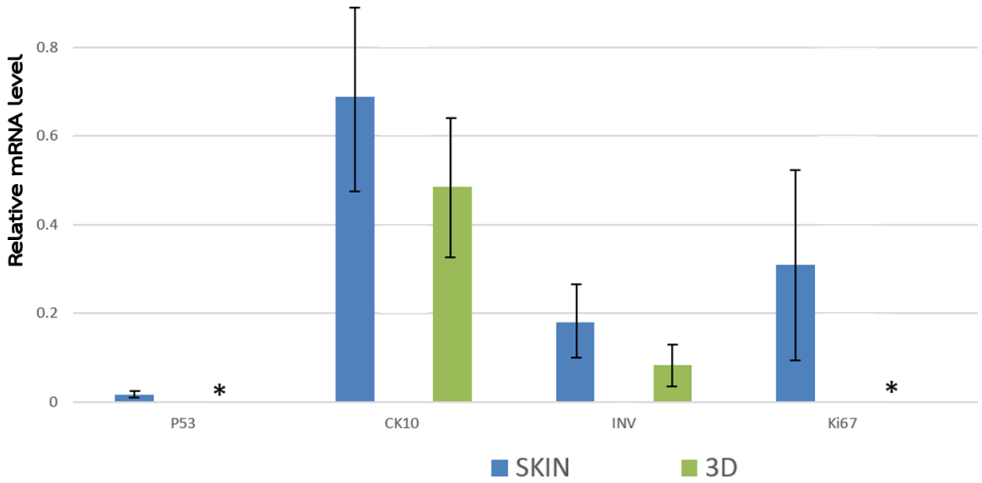

Figure 16 The histogram of mRNA expression of INV, p53, CK10, Ki67 in the normal skin (n=6) and 3D cultured CPEK (n=6). There were no statistical difference of INV and CK10 between 3D cultured CPEK and normal skin. The negative results of p53 and ki67 in 3D cultured CPEK was demonstrated. Normalized by β-actin. (mean+SD). Symbol * showed statistically significant differences (*p<0.05*).

Phase III: Alteration of canine cultured keratinocyte with INV gene silencing and treated cultured cell under UVB irradiation

3.1 siRNA

siRNA were designed by online program follow as Custom siRNA Design Tool User Guide of Bioneer pacific (https://us.bioneer.com/sirna/custom-sirna-ex.aspx). The designed siRNA for INV were in 3 set INV-1, INV-2 and INV-3. The experiment was performed in culture CPEK. The treatment of cultured CPEK with Involucrin-1 siRNA for INV knockdown showed a better result in reduction of mRNA levels of INV than Involucrin-2, Involucrin-3, and pool siRNA (Figure 17). The titration models

showed that 300 pmol was the lowest possible concentration of Involucrin-1 siRNA with the highly effective and minimize cytoxicity (Figure 18). Time of exposure, the deceasing expression of INV occured at 0 h to 48 h. At 48 h harvested cells shown slightly increasing INV expression (Figure 19). By LIVE/DEAD® staining, the 600 pmol siRNA concentration group were showed dead cell (red color) more than other concentrations, but not more than 20% (Figure 20). No distinctly different of LIVE/DEAD cell staining in variation of each time exposure.

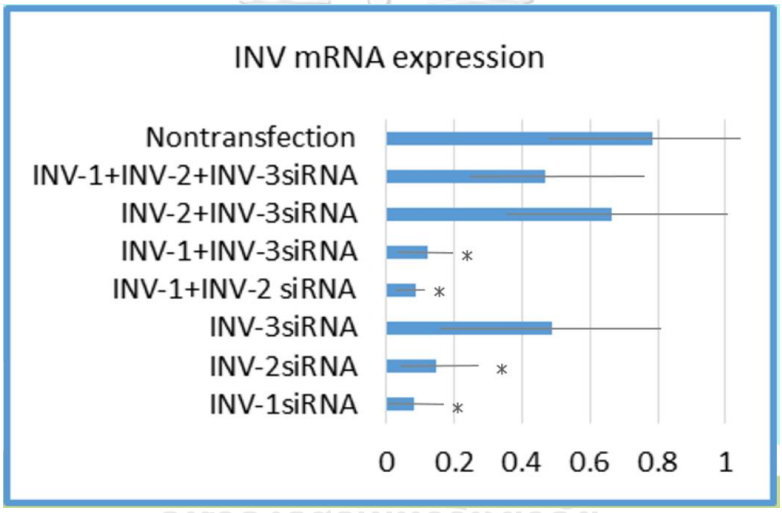

Figure 17 Demonstration of the silencing efficacy of 3 stranded custom designed of siRNAs targeting the Involucrin (INV) expression in cultured CPEK. INV mRNA levels were measured by qRT-PCR and shown base on delta Ct calculation. The bars were calculated from mean values of 3 replications. The single siRNA of INV-1 showed the best resulted on knockdown INV expression than others. Symbol * showed statistically significant differences ($p<0.05$).

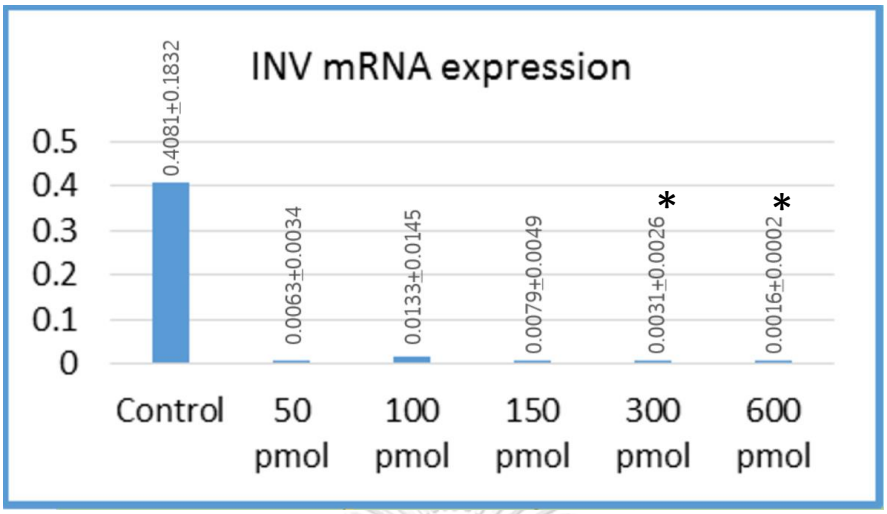

Figure 18 Optimization of INV-1 siRNA concentration in cultured CPEK. At 300 pmol concentration shown the best resulted on knockdown INV expression than others. Symbol * showed statistically significant differences (*p<0.05*).

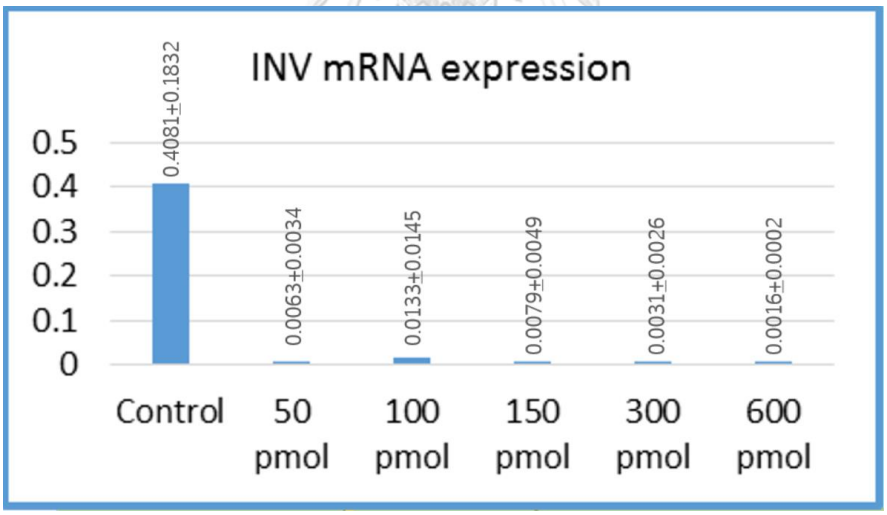

Figure 19 histogram of the INV expression knockdown times of 300 pmol INV-1 siRNA effects on Involucrin expression in cultured CPEK. INV expression was knockdown at 0 to 48 h after transfection. Symbol * showed statistically significant differences (*p<0.05*).

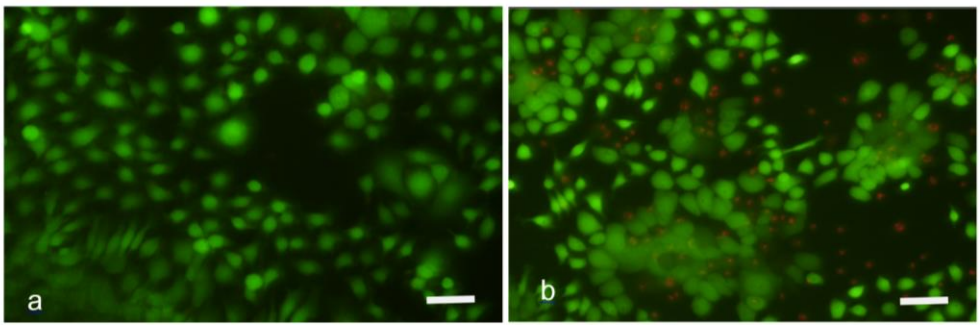

Figure 20 The LIVE/DEAD® cell viability/cytotoxicity test. Live cells fluoresce in bright green, whereas dead cells with compromised membranes fluorescence red-orange. 300 pmol, INV-1 siRNA transfected cell culture showed almost cell viability (a). 600 pmol INV-1 siRNA transfected cell culture shown dead cell 20% approximately (b). Leica DFC7000T with LAS X imaging software, IFC Bar=20 μm

3.2 UVB-irradiation

3.2.1 Morphology observation and immunofluorescence characterizations

The cytologic appearance of cultured CPEK were initially detected after 300 mJ/cm^2 UVB irradiation exposure, at 6 h. On observation, the cultured control cell which siRNA transfected without UVB irradiation appeared growing well and adhered the plate with perform regularly differentiation (Figure 21a-b). The evaluation of cell viability by LIVE/DEAD® cell viability test, there were a few dead cells in red color at 6 h. When compared with cultured cell which siRNA transfected with UVB irradiation at 6 h, the cell viability test showed a number of dead and sloughing cells in red color more than the control group.

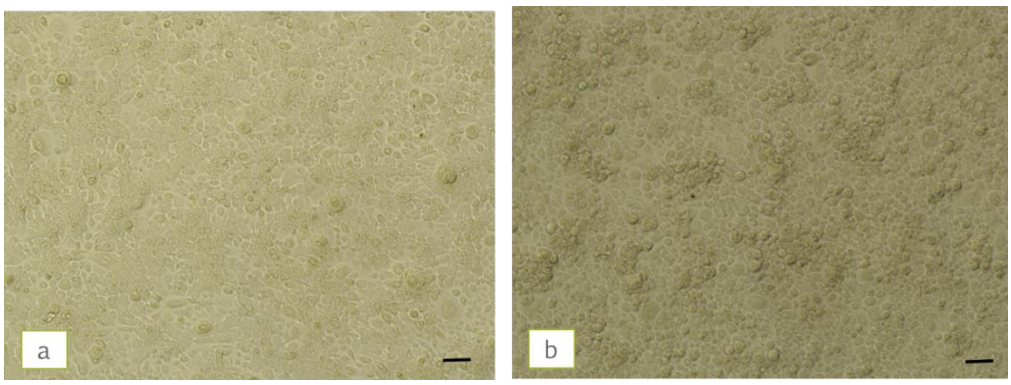

Figure 21 The cytologic appearance of cultured CPEK which siRNA transfected without UVB irradiation (a) appeared more growing and adhered the plate than cultured cell which siRNA transfected with 300 mJ/cm^2 UVB irradiation at 6 h (b). Phase contrast microscope (Zeiss® microscope) with Moticam 3.0mp digital camera, Bar=10 μm.

3.2.2 mRNA expression

The mRNA quantification of wild type p53 of cultured cell revealed an increase after 6 h exposure of 300 mJ/cm^2 of UVB and gradually increases the expression when increasing the exposure time respectively. After cell harvested at 24 h, the results of p53 expression were statistical significant difference increased in cultured cell with and without siRNA transfected under exposed UVB irradiation group than the group without UVB irradiation groups ($p<0.05$). There was no statistical difference of INV, CK10, and Ki67 expression between the groups with and without UVB irradiation (Figure 22).

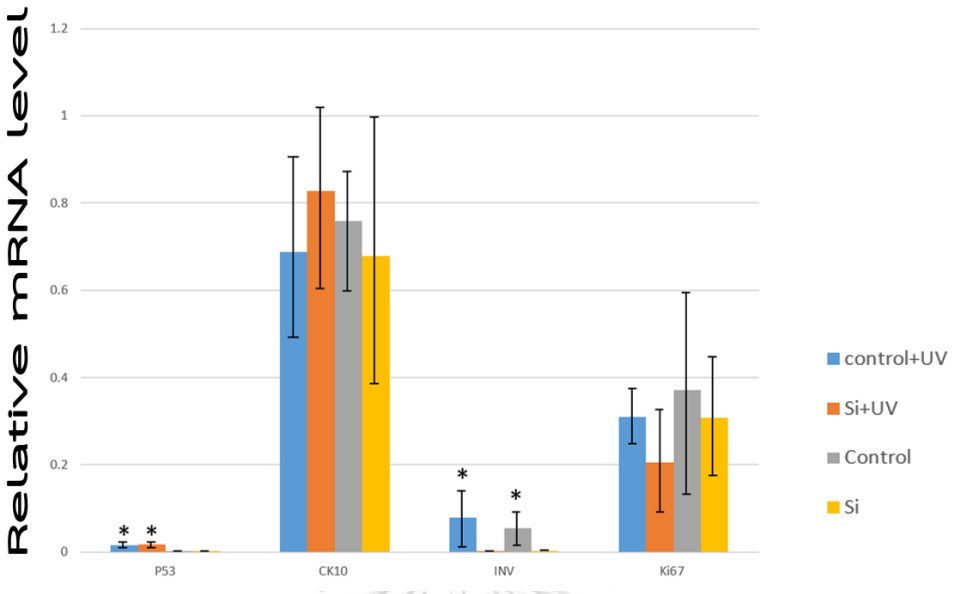

Figure 22 The histogram of mRNA expression score of INV, CK10, KI67 and p53 among the experiment groups (n=6); control+UV= control group with after 24 h exposure of 300 mJ/cm^2 of UVB irradiation, Si+UV= cultured cell which siRNA with UVB irradiation, Control= control culture cells without UVB irradiation, Si= cultured cell which siRNA without UVB irradiation. Normalized by β-actin. (mean+SD). Symbol * showed statistically significant differences (*p<0.05*).

CHAPTER V

DISCUSSION AND CONCLUSION

Incidence of skin tumor in Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University reveals 31.5% out of all the tumor case diagnosis. Majority of skin tumor were mast cell tumor (39.3%), SCC (22.5%), histocytoma (9.2%), melanoma (9%), sebaceous gland adenoma (6.2%), perianal gland adenoma (6%), and basal cell tumor (2.5%) (Assawawongkasem et al., 2016; Sailasuta, 2013). The samples in this study were collecting from 6 healthy dogs (1 males, 5 females) and 6 spontaneous cutaneous SCC (CSCC) patient dogs (2 males, 4 females) which were obtained by surgical biopsy from the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University and private animal clinics in Bangkok. The CSCC samples were collected from the dog at 9.5+3.5 of age which is similar to the previous report that age of affected dogs were 10.68+3.17 years (Assawawongkasem et al., 2016).

Due to the dog's owner did not allow to collect the sample of normal skin and the CSCC in the same dog. In this study, the clinically normal dog, healthy and no skin proplem, which requested for sterilization, were decided for collecting the normal skin in various part of the body. Which the dog's owners agreed to sign the consent form. Regard to the breed of dog, they were varied from case by case. Which it has been, recently demonstrated that cornified envelopes (involucrin and filaggrin), keratins (keratin 10, 5), and their mRNA expression among various breeds and between sexes of normal dog skin

were similarly in staining pattern. Thus, all breed could be used for normal dog skin samples (Theerawatanasirikul et al., 2012b).

All the skin samples both normal and CSCC, in case of normal skin showed no skin lesion and disease In addition, all the samples were screened for papilloma virus free sample by qPCR (Waropastrakul et al., 2012). Since, the major risk factor of CSCC, similar to human which, is induced by solar or UV radiation (DNA damaging agent). For minor effect, papilloma virus DNA has also been detected in 15% of CSCC dogs and chronic dermatosis, which abnormalities in molecular and genetic events has already been discussed (Blackwood, 2011; Chandrashekaraiah et al., 2011; Warland, 2011; Webb, 2009).

In order to compare the protein expression by IFC and mRNA expression of normal skin and CSCC revealed that in normal skin, INV, and cytokeratin 10 (CK10) were positive in intra-cytoplasmic pattern in whole layer of nucleated epidermis, the intra-nuclear staining of epidermal cells were positive Ki67, but was negative for p53 in all samples. While in CSCC, CK10 showed positivity pattern in similar to normal skin. For INV were positive weakly in same pattern as CK10. The intranuclear pattern were obvious positive with Ki67 and p53 more than normal skin. The appearance of protein expression staining pattern were corresponded to mRNA quantitation by qRT-PCR. The mRNA expression of INV of normal skin is more than SCC while Ki67 in normal skin less than CSCC that agreed to our preview study which had been demonstrated that the INV immunohistochemical staining in almost cutaneous neoplasm in dogs decreased significantly, compared to normal skin ($p<0.001$), Moreover, in CSCC groups were significantly inverse correlation of INV with proliferative index, Ki67 and histology grading ($p<0.0001$) (Assawawongkasem et al., 2016). It is implied that the defective terminal differentiation of transformed stratified squamous epithelial cells has been recognized by the

decreased of INV expression with many unknown mechanisms. However, INV had been further studied in human dermatology and medical research about its normal epidermal renewal structures, patterns and functions, including various immune disease and genetic defect of skin lesion (Bernard et al., 1986; Hertle et al., 1992; Jensen et al., 2004). Currently, it has been proved that in human tumor model that INV is an oxygen-regulated protein, a useful endogenous marker hypoxia (Chou et al., 2004)and significantly correlated with clinicopathologic sign of SCC patients (Nozoe et al., 2006).

The role of cytokeratin in this study, the CK expression has been employed to compare the potential of INV as a tumor marker of skin tumor in dog. Generally, CK is a differentiated keratinocyte marker (Shibata et al., 2008). The CK10, in this study was demonstrated in normal skin and increased its appearance in CSCC which was difference to INV that was deceased. Recently, it has been reported on the expression of CK in epidermal neoplasm and usually conserved during malignant transformation of the epidermis, which other markers that identifying the cell of epidermal origin may be lost. Thus, the identification of CK in poorly differentiated tumor could help to establish the epithelial tumor origin of the malignancy (Watanabe et al., 1995). In this study, CK10 is a high molecular weight keratin that normally expresses in the suprabasal layer of the epidermis or keratinizing stratified epithelia and in differentiated areas of highly differentiated squamous cell carcinomas (Basta-Juzbasic et al., 2004).

In this study, the CSCC samples, INV showed decreasing and Ki67 showed increasing significantly. The relationship of Ki67 and INV expression had been reported in the proliferative, pre-neoplastic and neoplastic skin in human (Caldwell et al., 1997). Additionally, it was found that related to rapid growth and recurrence in non-melanotic skin cancer (NMSC) (Martins et al., 2009;

Nozoe et al., 2006) and poor prognosis in canine SCCs (Assawawongkasem et al., 2016).

The expression of wild type p53 in CSCC tended to increasing when compared to normal skin. The experiment had decided to study the wild type p53 in the cultured cells in a limited time. The protein expression by IFC for wild type p53 was negative in normal skin and low expression in CSCC. Which possibly due to the wild-type p53 protein has extremely short half-life and is could be found in small quantities inside cells or cannot be detected in normal cells by using immunohistochemistry (Dabbs, 2013). In cancerous cell, the occurring of mutant p53 protein can also inhibit wild type p53 of itself (Blagosklonny, 2002). However, the mRNA expression of wild type p53 could be detected in CSCC more than normal skin. It is suggested that p53 protein level is low in normal cell, which agreed to the previous report (Blagosklonny, 2002).

The development of cultured keratinocytes either from cell line (CPEK) or from dog skin isolation, using a primary explant technic was performed. In this study, the cultured CPEK cell by using Cnt-09 media was successfully conducted by producing 80-90% confluent appearance after 3-4 day. However, it was found that by using the same media (CnT-09) for the primary cell culture both normal skin and CSCC, the good results could not be obtained. That could be due to the condition of culture and growth requirement that keratinocyte requires intensively condition on reaching confluence in low density cells, and slow growing when compared to other cell types. Therefore, special conditions should be used to promote their attachment and preserve their differentiate status (Freshney, 2006). The CnT-09 media which promote proliferation is suitable for pure keratinocyte cell line as in our experiment. In case of primary cell culture. There is other cell

contaminating especially, fibroblast. That is preventing the keratinocyte growth and inducing keratinocyte's differentiation. The cell was undergone degeneration rapidly. Moreover, the using of antibiotic/antimicrobial in washing processes of primary cell preparation also affect to keratinocyte culture (Freshney, 2006). In this experiment, the primary culture of skin could not be obtained, possibly due to media. As previous described in human keratinocyte culturing should be based on serum-free with low calcium culture media because some growth factors in serum such as transforming growth factor ß (TGF- ß) inhibited epithelial proliferation and differentiation(Freshney, 2006). It is suggested that the technical problems such as contamination and additional media factor that should be improved for the primary cell culture of skin and CSCC. The additional factors such as BSA, cholera toxin and murine EGF are necessary using to promote growth and differentiation of neoplastic canine keratinocyte (Crameri et al., 1997).

The 3D cultured CPEK and proliferation in air-liquid interface method have been conducted in this experiment that help the cell's proliferation. The previous experiments showed that air exposure is an important factor on the proliferation, differentiation of keratinocytes. It is possible on providing an experimental model in animal (Inoue et al., 2001). The cultured CPEK developed a monolayer in 2 days without antibiotic/antimycotic at 37°c in 5% CO_2 atmophere which is practically. The 3D cultured CPEK were conducted for 14 days. At day 14, the cells revealed the proliferation and differentiation appearances in the epidermal characteristic similar to the epidermis. When, the culture continued until 21 days, it was found that the cells ceased and starting to degenerate. Which is in corresponded to the previous study that develop canine epidermis equivalent by interface between epidermal keratinocyte and fibroblast embedded into biomatrix collagen gel and the skin

equivalent were observed in vitro up to 3 weeks, with optimal growth period of 2 weeks (Serra et al., 2007; Yagihara et al., 2011) and when using 3D cultured keratinocyte from foot pad with collagen gel could be developed in 5 days (Yamazoe et al., 2007). Thus, the 3D cultured CPEK under air-liquid interface technique was successfully developed the artificial epidermis in dog in 14 days. The 3D culture's life time is shorter than dog epidermis that has a turnover rate of 22 days and approximately 28 days in human (Yager, 1991).

3D cultured CPEK, the protein expression by IFC and mRNA expression of INV and CK10 revealed positive results while ki67 and p53 showed negative which are in the similar patterns of normal skin. In comparison to mRNA expression of normal skin and CSCC, there were no statistically difference of INV, CK10 between 3D cultured CPEK and normal skin. It could be demonstrated that 3D cultured CPEK is quite similar to normal skin in both histology and gene expression. Which, the expression of INV and CK10 revealed the differentiation status of the keratinocytes.

On the contrary, the negative results of Ki67 and p53 mRNA expression might be by using the 3D cultured CPEK which these cells ceased on their proliferation and proceeded completely differentiation. For previous report, the Ki67 showed weak positive staining at the bottom most layer of the cultured CPEK in collagen gel (Yagihara et al., 2011). The difference result might be by the system of cell culture as air-liquid interface technic and feed layer. The result of wild type p53, which had been reported that p53 demonstrating on primary human keratinocyte culture but its levels were affected by various growth conditions. That will be an impact on cellular adhesion and its differentiation (Nigro et al., 1997).

In this study, the experiment has been conducted by the designed siRNA from dog INV in our laboratory. It has been known that the commercial

siRNA are all for human and rat, mice available in the market which is difference from dog. Therefore, the siRNA was designed by online program follow as custom siRNA Design Tool User Guide of Bioneer pacific (https://us.bioneer.com/sirna/custom-sirna-ex.aspx). The top 3 sets of designed siRNA; INV-1, INV-2, INV-3 has been selected to the preliminary test in monolayer cultured CPEK and were titrated for the lowest concentration that could knockdown mRNA of INV with minimum cytotoxic effect. The results showed that 300 pmol was the lowest possible concentration of Involucrin-1 siRNA with the highly effective and minimium cytoxicity. Regards to the exposure time, the decrease of INV expression was approximately or more than 70% occurring at 0 h to 48 h. At 48 h the harvested cells showed slightly increasing of INV expression. That is the period of knockdown in our experiment. explained that their experiments that a single transfection siRNAs achieved in cancer cells more than 80% knockdown that lasted 5–7 days post-transfection (Li, 2008). In addition, the higher siRNA concentrations did not show the result in stronger or long-lasting knockdown, but are likely to cause off-target effects (Fakhr et al., 2016; Reynolds et al., 2004). In some cases, the repeated siRNA transfection resulted in the improvement to knockdown during select timepoints post-transfection of the duration of gene silencing by siRNA method. Which was depended on the designed siRNA and transfection method. However, in our study, the custom designed INV siRNA in dog could be further developed in the percentage of knockdown and duration of gene silencing. The concentration of INV-1 siRNA at 300pmol, has been decided to be the lowest concentration that was suitable to knockdown INV with minimum cytotoxicity. The cultured 3D keratinocyte could not be developed by using siRNA-INV transfected. These results might be due to the effect of transfection process that destroyed the adhesion functions of keratinocyte or

INV lost which it is an important cornified envelope precursor protein (Eckert et al., 1997; Rice and Green, 1977). Therefore, the monolayer of CPEK were suitable to apply UVB irradiation.

In this study, UVB was used, which is a well-known cause of cutaneous neoplasia. The radiation produces a dimer of these molecules that can lead to mutation when they are repaired incorrectly (Cullen, 2002). The importance of genetic injury in the pathogenesis of skin cancer is supported by the presence of mutations in the dipyrimidine regions of the ras oncogene and p53 tumor suppressor gene in both human and mice following UVB exposure (Cullen, 2002). The effect of UVB irradiation to cultured CPEK was demonstrated by the increasing of wild type p53 mRNA. The p53 expression is a sensitive marker for UVB reactivity (Will et al., 2000). Therefore, wild type p53 expression could mediate its DNA damage responses by modulating cellular transcription as well as exhibits various other biochemical activities. That are directly related to its function as a major control element in preserving the integrity of the cell's genetic information. The results showed that 300 mJ/cm^2 UVB irradiation exposure could be induce changing in 6 h. after UVB exposed in with and without siRNA transfected cell. Whereby, the cell viability test showed a number of dead cells, sloughing cells and mRNA quantitation of wild type p53 of cultured cell revealed an increase when compared to the control groups. There was no statistical difference of INV, CK10, and Ki67 expression between the groups with and without UVB irradiation.

From the obtained results it could be explained that the decreasing of INV in canine keratinocyte did not effect on pathogenesis of cultured keratinocyte induced by 300 mJ/cm^2 of UVB irradiation. The wild type p53 expressuon is a suitable marker for this study, which p53 were responded by UVB stress after 24 h exposure agreed to the previous experiments (Will et al.,

2000). In addition, the cultured human keratinocytes experiment had been shown that p53 responded in a UVB dose and time dependent manner and can be influenced by a variety of factors such as cellular adhesion or the state of differentiation (Kumar et al., 1999). The expression of Ki67 in this study was not significantly changed after UVB exposed which might be, the Ki67 expression of cultured CPEK need exposure time or must be using more than 24 h for induce Ki67 mRNA expression that should be done in future investigation.

In conclusion, the obtained results demonstrated that the protein expression staining pattern by IFC and the level of mRNA expression of INV are difference between normal skin and CSCC in dog which are all papilloma virus free samples. The cultured 3D CPEK in air liquid interface system has been successfully developed as an artificial epidermis in dog by histology and its INV and CK10 protein and mRNA expression similarly to normal skin biopsy. Finally, this is the first report on the design of siRNA transfected in the monolayer cultured CPEK for INV's knockdown in dog. It is suggested that INV doesn't effect on the induced by 300 mJ/cm^2 of UVB irradiation, which UVB is the major cultured keratinocyte cause of CSCC in dog.

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