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กองทุนรัชดาภิเษกสมโภช

รายงานผลการวิจัย

การศึกษาการแสดงออกของยีนอีแคทฮีริน, ซินดีแคน 1, เมทริกเมทัลโลโปรติเนส-2, -7, -9, -14 และตัวยับยั้งฤทธิ์ของเอ็มเอ็มพี-1 และ -2 ในโรคมะเร็งเม็ดสีในช่องปากสุนัข

โดย

ผู้ช่วยศาสตราจารย์สัตวแพทย์หญิง ดร.กรรณาภรณ์ สุริยผล

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การคัดเลือกจีนอ้างอิงสำหรับการทำปฏิกิริยาลูกโซ่พอลิเมอเรสเรียลไทม์ในเนื้องอกในช่องปากสุนัขชนิดธรรมดาและมะเร็ง

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บทคัดย่อ

การศึกษาวิจัยครั้งนี้มีวัตถุประสงค์เพื่อ 1.) คัดเลือกยีนอ้างอิงที่เหมาะสมเพื่อใช้ในการทำปฏิกิริยาลูกโซ่พอลิเมอเรสแบบเรียลไทม์ย้อนกลับในโรคมะเร็งช่องปากสุนัข ประกอบด้วยโรคมะเร็งเม็ดสีในช่องปาก (หรือโรคมะเร็งช่องปากเมลาโนมาในสุนัข) และโรคมะเร็งช่องปากสะความัสเซลล์ ซึ่งทั้ง 2 โรคเป็นโรคมะเร็งช่องปากที่พบได้บ่อยที่สุด 2.) ศึกษาการแสดงออกของยีนอีแคทฮีริน (*CDH1*), ซินดีแคน 1 (*SDC1*), เมทริกเมทัลโลโปรติเนส-2, -7, -9, -14 (*MMP2, MMP7, MMP9, MMP14*) และตัวยับยั้งฤทธิ์ของเอ็มเอ็มพี-1 และ -2 (*TIMP1* และ *TIMP2*) ในระดับอาร์เอ็นเอและศึกษาการแสดงออกของโปรตีน CDH1 SDC1และ Ki-67 โดยวิธีอิมมูโนฮีสโตเคมีในสุนัขที่เป็นโรคมะเร็งช่องปากเมลาโนมาเปรียบเทียบกับสุนัขปกติ และ 3.) ศึกษาความสัมพันธ์ระหว่างการแสดงออกของยีนและระดับความรุนแรงของโรคมะเร็งช่องปากเมลาโนมา โดยทำการเก็บตัวอย่างชิ้นเนื้อมะเร็งช่องปากเมลาโนมาจากสุนัขป่วยระยะโรคต่างๆ รวม 12 ตัวอย่าง โดยทำการแบ่งกลุ่มชิ้นเนื้อตามระยะโรค (TNM stage) ซึ่งแสดงความรุนแรงของโรคเป็นระยะแรก (ระยะ 1-2) 4 ตัวอย่างและระยะท้าย (ระยะ 3-4) 8 ตัวอย่าง ชิ้นเนื้อมะเร็งช่องปากสะความัสเซลล์ 7 ตัวอย่าง ชิ้นเนื้อเหงือกปกติ 8 ตัวอย่าง ทำการคัดเลือกยีนอ้างอิงที่เหมาะสมจากยีนอ้างอิงที่ใช้ทั่วไปจำนวน 6 ยีน ได้แก่เบตาแอคติน (*ACTB*), เบตาทูไมโครกลอบูลิน (*B2M*), กลีเซอรอลดีไฮด์ธรีฟอสเฟตดีไฮโดรจีเนส (*GAPDH*), ไรโบโซมอลโปรตีน L13a (*RPL13a*), ไรโบโซมอลโปรตีน S5 (*RPS5*) และไรโบโซมอลโปรตีน S19 (*RPS19*) โดยใช้อัลกอริทึมต่างๆ 5 วิธีในการประเมินยีนอ้างอิง จากนั้นได้ใช้ยีนอ้างอิงที่ได้รับการคัดเลือกในการ

นอมัลไลซ์การแสดงออกของยีนที่สนใจ พบว่า *ACTB, RPS5* และ *RPS19 เป็น*ยีนอ้างอิงที่เหมาะสมในโรคมะเร็งช่องปากทั้ง 2 โรค และเมื่อทำการเปรียบเทียบการแสดงออกของยีนจากตัวอย่างชิ้นเนื้อสุนัขป่วยเป็นโรคมะเร็งช่องปากเมลาโนมาในระยะต่างๆ กับชิ้นเนื้อปกติ ในภาพรวมพบการแสดงออกของยีน *CDH1* และ *SDC1* ลดลงทั้งในระดับเอ็มอาร์เอ็นเอและโปรตีน ทั้งสองยีนจะแสดงออกเป็นโปรตีนที่มีหน้าที่กดการเกิดเนื้องอก (tumor suppressor gene) และ พบการแสดงออกของยีน *MMP2* และ *MMP14* เพิ่มขึ้น โดย MMP14 เป็นตัวช่วยในการสร้าง proMMP2 ซึ่งแสดงถึงการลุกลามของโรคมะเร็ง นอกจากนี้ยังพบการแสดงออกของยีน *MMP7* ลดลง เมื่อแยกระยะพบว่าในระยะแรกและระยะท้ายมีการแสดงออกของยีน *TIMP1* และ *TIMP2* เพิ่มขึ้นตามลำดับ TIMP จะออกฤทธิ์ยับยั้งการแสดงออกของยีน *MMP* และยับยั้งการแพร่กระจายของเซลล์มะเร็ง สำหรับการแสดงออกของโปรตีน Ki-67 ซึ่งแสดงการเพิ่มจำนวนเซลล์ พบการแสดงออกมากขึ้นในสุนัขป่วยเมื่อเทียบกับสุนัขปกติ โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นว่า *ACTB, RPS5* และ *RPS19 เป็น*ยีนอ้างอิงที่เหมาะสมสำหรับโรคมะเร็งช่องปากเมลาโนมาและโรคมะเร็งสะความัสเซลล์ ในโรคมะเร็งช่องปากเมลาโนมาพบการแสดงออกของยีนกดการเกิดเนื้องอกลดลงซึ่งสอดคล้องกับการแสดงออกของยีน *MMP2* และ *MMP14* ซึ่งแสดงถึงการเกิดโรคมะเร็ง นอกจากนี้เนื่องจากในระยะแรกและระยะท้ายของโรคพบการแสดงออกของยีนที่มีหน้าที่ยับยั้งการลุกลาม/แพร่กระจายของโรค ได้แก่ *TIMP1* และ *TIMP2* ตามลำดับ จึงควรมีการศึกษาถึงการนำยีนเหล่านี้มาใช้แสดงพยาธิสภาพและการวินิจฉัยโรคในระยะต่างๆ ต่อไป

Project Title Study the gene expression of E-cadherin, syndecan1, matrix metalloproteinases-2, -7, -9, -14 and tissue inhibitors of metalloproteinases-1 and -2 in canine oral melanoma

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Selection of Reference Genes for Real-time Polymerase Chain Reaction in Canine Oral Tumor and Cancer

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Abstract

The objectives of this study were to 1.) select the suitable reference genes for quantitative real-time polymerse chain reaction in the most common canine oral cancers: oral melanoma (OM) and oral squamous cell carcinoma (OSCC), 2.) study the gene expression of E-cadherin (*CDH1*), syndecan 1 (*SDC1*), matrix metalloproteinases-2, -7, -9, -14 (*MMP2, MMP7, MMP9, MMP14*) and tissue inhibitors of metalloproteinases-1 and -2 (*TIMP1, TIMP2*) in canine OM at the mRNA level and

study the CDH1, SDC1 and Ki-67 protein expression by immunohistochemistry, and 3.) study the association of gene expression and the tumor, node, metastasis (TNM) stage. Twelve OM tissues with different TNM stages together with 7 OSCC and 8 normal gingival tissues were collected for reference gene selection. Five algorithms were used to evaluate 6 candidate reference genes, including beta actin (*ACTB*), beta-2-microglobulin (*B2M*), glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L13a (*RPL13a*), ribosomal protein S5 (*RPS5*), and ribosomal protein S19 (*RPS19*). The result showed that the cohort of the most suitable reference genes for canine OM and OSCC were *ACTB*, *RPS5* and *RPS19* and they were then used to normalize target genes in qRT-PCR. In OM, *CDH1* and *SDC1*, encoding tumor suppressor proteins, were found to be down-regulated, corresponding to the IHC results. *MMP7* gene was also found to be down-regulated whereas *MMP2* and *MMP14*, encoding the proMMP2 activator, were upregulated, indicating cancer progression. *TIMP1*, encoding an inhibitor of tumor progression and metastasis, and *TIMP2*, encoding an inhibitor of MMP2, were upregulated in the early stage (stages 1 and 2) and late stage (stages 3-4) of the disease, respectively. Ki-67 expression, an indicator of proliferating cells, was also found to be upregulated in OM. In conclusion, the present study reported the suitable reference genes for canine OM

and OSCC. The association of tumor suppressing gene and some MMP gene expression with OM was presented, regardless of the disease stages. Upregulation of *TIMPs* in the different stages of OM should be further studied for the possibility to be used as pathological and diagnostic markers of the disease stages.

Table of Contents

Acknowledgement......ii

บทคัดย่อ......iii

Abstract......v

List of Tables......ix

List of Figures......xi

1. Introduction......1

2. Survey of Related Literature......3

3. Procedures......10

4. Results......18

5. Discussion......29

6. Conclusion......32

7. Suggestion for Further Work......33

8. References......33

List of Tables

1. Primers used in the present study 15

2. TNM and histopathological classifications of 13 dogs with oral malignant melanoma 20

3. Ranking of the reference genes in canine oral melanoma and oral squamous cell carcinoma from high to low stability by various algorithms 21

4. Ranking of the reference genes in canine oral melanoma from high to low stability by various algorithms 22

5. Ranking of the reference genes in canine oral squamous cell carcinoma from high to low stability by various algorithms 22

6. Ranking of the reference genes in canine oral melanoma and/or oral squamous cell carcinoma from high to low stability with geNorm and NormFinder programs, calculated by the web-based program. 23

7. Expression ratios of *CDH1* (upper row) and *SDC1* (lower row) against several reference genes in the oral melanoma and/or oral squamous cell carcinoma 25

8. Expression ratios of *CDH1, SDC1, MMP2*, *MMP7*, *MMP9*, *MMP14*, *TIMP1* and *TIMP2* against 3 reference genes, *ACTB*, *RPS5* and *RPS19*, in the oral melanoma at different stages 26

9. Immunohistochemical scores for Ki-67 (as percent positive nuclei), and for E-cadherin and syndecan1 (as percent positive area) in canine oral melanoma at various TNM stages 29

List of Figures

1. Histopathology of melanotic melanoma, amelanotic melanoma and squamous cell carcinoma......19
2. Immunohistochemical staining for Ki-67 in canine oral melanoma. Ki-67 expression in normal canine gingiva, amelanotic melanoma and melanotic melanoma ...27
3. Immunohistochemical staining for E-cadherin in canine oral melanoma. E-cadherin expression in normal canine gingiva, amelanotic melanoma and melanotic melanoma 28
4. Immunohistochemical staining for syndecan1 in canine oral melanoma. Syndecan1 expression in normal canine gingiva, amelanotic melanoma and melanotic melanoma 28

1. Introduction and Aim

Oral cavity is a common site for canine cancers. The most frequent canine oral cancers are oral melanoma (OM) and non-tonsillar oral squamous cell carcinoma (OSCC). Studying of gene expression associated with oral mass by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is currently increasing. In order to obtain reliable gene expression results, the normalization with reference genes is required. However, reference gene selection for canine oral tumors has not been performed. The first objective of the present study aimed to identify the potential reference genes in OM and OSCC. The suitability of 6 candidate reference genes, beta-actin (*ACTB*), beta-2-microglobulin (*B2M*), glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), ribosomal subunit L13a (*RPL13a*), ribosomal protein S5 (*RPS5*) and ribosomal protein S19 (RPS19) was evaluated. Five algorithms, geNorm, Normfinder, BestKeeper, the comparative delta Ct method and RefFinder, were employed to analyze the expression stability. On a side note, OM has been routinely considered an extremely malignant tumor with a high degree of local invasiveness and high metastatic propensity. The World Health Organization (WHO) has defined clinical staging scheme for dogs with OM based on size and metastasis. The proteolytic degradation of extracellular matrix (ECM) that surrounds tumor cells is an

essential step in tumor invasion and the development of metastasis. The process is performed by a group of matrix metalloproteinases (MMPs). Balanced functions of MMPs and their tissue inhibitor matrix metalloproteinases (TIMPs) are important for extracellular matrix homeostasis. In addition, various specific cell-MMP interactions have been reported. The loss of surface glycoproteins such as E-cadherin (CDH1) and syndecan 1 (SDC1) and the overexpression of MMPs are common features of an invasive tumor. Hence, the second objective of the present study aimed to investigate the expression of molecular markers, *CDH1*, *SDC1*, *MMP2*, *MMP7*, *MMP9*, *MMP14*, *TIMP1* and *TIMP2*, in OM by qRT-PCR and by immunohistochemistry (IHC) (for CDH1 and SDC1). The correlation of the expression of molecular markers including the proliferation index (Ki-67) to WHO staging scheme was also investigated.

Aims

1. To identify the potential reference genes in OM and OSCC

2. To investigate the expression of molecular markers, *CDH1*, *SDC1*, *MMP2*, *MMP7*, *MMP9*, *MMP14*, *TIMP1* and *TIMP2*, in OM by qRT-PCR and by immunohistochemistry (IHC) (for CDH1 and SDC1)

Research questions

1. What were the suitable reference genes for qRT-PCR normalization in canine oral melanoma and non-tonsillar oral squamous cell carcinoma?

2. Did the expression of *CDH1*, *SDC1*, *MMP2*, *MMP7*, *MMP9*, *MMP14*, *TIMP1* and *TIMP2* associate with oral melanoma?

2. Survey of Related Literature

Canine oral melanoma and non-tonsillar oral squamous cell carcinoma

Canine oral cancers account for 6-7% of all cancers in dogs (Liptak and Withrow 2013) with approximate 44% and 31% of all oral malignancies being OM and OSCC, respectively (Priester and McKay 1980). Non-tonsillar OSCC has high incidence with 50-78% of all OSCC (Brooks et al 1998). Both OM and non-tonsillar OSCC aggressively invade adjacent tissues. OM is frequently seen in Scottish terriers, golden retrievers, poodles, dachshunds, chow chow, pekingese/poodle mix breeds (Goldschmidt 1985; Hahn et al 1994; Ramos-Vara et al 2000). OM is found in the following locations by order of decreasing frequency: gingiva, lips, tongue, and hard palate. Melanoma cells are normally pigmented, however, amelanotic oral melanomas, lacking pigmentation granules and being pink, have been reported (Choi and Kusewitt 2003; Smedley et al 2011). Canine OM is very similar to human OM.

Both are locally aggressive, with a rapid growth and tendency to spread to local lymph nodes and lungs (Porrello et al 2006). WHO has defined clinical staging scheme for dogs with OM based on tumor size and metastasis as stage I = < 2 cm. diameter tumor, stage II = 2 cm. to < 4 cm. diameter tumor, stage III = 4 cm. or greater tumor and/or lymph node metastasis and stage IV = distant metastasis (Bergman 2007). For OSCC, histological grading is widely used to help prognosis and grades I-III correlate to well, moderately and poorly differentiated, according to the pleomorphism, mitotic activity and keratinization (Nemec et al 2012).

Reference gene selection for quantitative real-time polymerase chain reaction

Since several studies on the gene expression profiles of the canine melanoma were demonstrated by qRT-PCR, the normalization to reference genes is required to remove any technical variation during the qRT-PCR processes (Radonić et al 2004). According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, the optimal number of reference genes must be experimentally determined and the statistical algorithms used to identify the best reference genes have to be reported (Bustin et al 2009). Several reference genes were commonly used in canine gene expression study, including beta-actin (*ACTB*) (Peleg et al 2010), beta-2-microglobulin (*B2M*) (Brinkhof et al 2006), glyceraldehyde-3-

phosphate dehydrogenase (*GAPDH*) (Park et al 2013), ribosomal subunit L13a (*RPL13a*) (Peters et al 2007), ribosomal protein S5 (*RPS5*) and ribosomal protein S19 (*RPS19*) (Schlotter et al 2009; Theerawatanasirikul et al 2012). However, only a few publications of potential reference genes in canine cancers have been reported (Etschmann et al 2006; Tsai and Breen 2012; Zornhagen et al 2014), not including canine oral cancers.

Several algorithms were established to measure the expression variation of candidate reference genes such as geNorm (Vandesompele et al 2002), NormFinder (Andersen et al 2004), BestKeeper (Pfaffl et al 2004), comparative delta threshold cycle (dCt) (Silver et al 2006) and RefFinder (Taki et al 2014). In the geNorm software, each Cq value was transformed into a deltaCq (dCq) and then taking the equation $2^{(-dCq)}$. Hence, all data is expressed relative to the expression of the least expressed gene. Then gene expression stability was defined as M value, the average pairwise variation of each gene compared to that of all other reference genes. M values are calculated from the standard deviation (SD) of the logarithmically transformed expression ratios. Genes with lower M values also have lower variations of expression. In addition, the program also provides the optimal number of reference genes from the pairwise variations ($V_{n/n+1}$) between combination of sequential

normalization factors (NF_n and NF_{n+1}). The NF calculation is based on the geometric means of several reference genes and NFs stepwise add gene numbers until $V_{n/n+1} \leq 0.15$ (Vandesompele et al 2002). NormFinder program uses the mathematical model to rank expression stability values from the measurement of the overall gene expression variation and the variation across subgroups of each gene (Andersen et al 2004). Each Cq value was log transformed (natural base (e) logarithm) before calculation. Bestkeeper ranks expression stability of reference genes according to their average SD values calculated from Ct or, according to the MIQE guidelines, quantification cycle (Cq). The BestKeeper Index, the geometric mean of Cq values of all candidate reference genes is used to estimate correlations among the reference genes and between each reference gene. BestKeeper can also analyze sample integrity as an InVar value (Pfaffl et al 2004). In the comparative dCt method, the expression stability of reference genes is ranked according to the average SD values of the difference of Cq values of each reference gene when does pairwise analysis with other reference genes in all samples tested (Silver et al 2006). RefFinder is a web-based comprehensive tool. It uses raw Cq values of each sample to search for the most stable gene selection online by comparison of the geNorm, NormFinder, BestKeeper and comparative dCt methods, and makes comprehensive gene stability

ranking by calculating weight to each gene and a geometric mean of their weights for all ranking genes is presented (Taki et al 2014).

The web-based program (http://www.leonxie.com/referencegene.php) was used to calculated stability values of RefFinder and other algorithms. The stability values of each algorithm calculated from the web-based program were compared to those calculated by the original software. For all algorithms, the highest stably expressed gene has the lowest stability values (M values, average SD values, etc.). Concordance from at least three algorithms was used to determine the most stably expressed reference gene to normalize target genes in OM and OSCC (Jacob et al 2013). The integrity of each sample was determined by InVar values, using the BestKeeper software. Strong deviating samples due to technical errors such as sample degradation and incomplete reverse transcription were discarded. The REST 2009 software with a pair-wise fixed reallocation randomization test was used to show significant differences of *CDH1* and *SDC1*expression between normal and cancerous tissues. Results with a P value < 0.05 were considered significant.

Ki-67

Ki-67 protein is a cellular marker for proliferation (Scholzen and Gerdes 2000). It is found in any active phase of the cell cycle (G1, S, G2, and mitosis), but not in

resting cells (G0) (Brown and Gatter 2002). The fraction of Ki-67-positive tumor cells (the Ki-67 index) has been demonstrated to be correlated with the clinical course of several cancers, including canine oral melanoma (Millanta et al 2002; Bergin 2011).

Matrix metalloproteinases (MMPs)

MMPs are zinc-dependent endopeptidases. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine/cytokine in/activation (Van Lint and Libert 2007). MMPs are also thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense. They also play an important role in tissue remodeling associated with various pathological processes such as cirrhosis, arthritis, and metastasis. MMP2 and its activation are thought to be important in the invasive and metastatic phenotypes of human melanomas (Hofmann et al 2000). All MMPs are synthesized in the inactive form (zymogen). A number of MMPs can cleave the prodomain of other MMP zymogens leading to activation and it has been suggested that they mediate the final proteolytic step to produce a fully active enzyme. MMP activity is further modulated through interactions with their natural inhibitors, the TIMPs, which can control MMP activation. ProMMP9 forms a tight complex with TIMP1 and TIMP3. And proMMP9–

TIMP complexes are potential inhibitors of metalloproteinases (Nagase et al 2006) whereas proMMP2 activation requires cooperative action of both MMP14 and low concentration of TIMP2. However, TIMP2 at high concentration can inhibit unbound active MMP2 (Goldberg et al 1992; Gomez et al 1997; Bernardo and Fridman 2003; Toth et al 2003). Imbalance between MMPs and TIMPs influence biologic aggressiveness of many types of cancer. Increased MMP expression and/or decreased TIMP expression favors proteolysis (Bramhall 1998; Butler et al 1998; McCawley and Matrisian 2000). Overexpression of TIMP1 in melanoma cells had been previously demonstrated to reduce metastasis (Khokha 1994).

E-cadherin and Syndecan 1

E-cadherin is a glycoprotein that is responsible for calcium-dependent intercellular adhesion by homotypic interaction. It plays an important role in intercellular adhesion in most epidermal layers except in the most superficial, terminally differentiated cells. Reduction or loss of E-cadherin has frequently been associated with cell-to-cell disengagement, tumor grade, invasion, and metastasis (Chen and Obrink 1991; Shiozaki et al 1996).

Syndecan 1, is a transmembrane heparan sulfate proteoglycan which is abundantly expressed by keratinocytes in most epidermal layers except in the most

superficial, terminally differentiated cells. The syndecan 1 protein functions as an integral membrane protein and participates in cell proliferation, cell migration and cell-matrix interactions via its receptor for extracellular matrix proteins. Proteolytic SDC1 cleavage has been attributed to a variety of enzymes, which include MMP7, MMP9, MMP14 (Endo et al 2003; Brule et al 2006; Chen et al 2009). In 3D co-cultures of immortalized human mammary fibroblasts and T47D breast carcinoma cells, SDC1 release from the cell surface is mediated by fibroblastderived membrane-type 1 matrix metalloprotease (MT1-MMP or MMP14), assigning another cancer-promoting function to this enzyme (Su et al 2008).

E-cadherin and syndecan 1 form a powerful invasion suppressor complex and loss of cell surface syndecan 1 causes transformation of epithelial cells (Kato et al 1995; Furukawa et al 1997). MMP7, the smallest member of the MMP family, can cleave and shed cell surface proteins such as syndecan 1 and E-cadherin in SCC (Noe et al 2001; Kivisaari et al 2008).

3. Procedures

Animals

Twelve OM tissues and 7 OSCC tissues were obtained from dogs submitted to surgery at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University (age range 10-16 years and 3-15 years, respectively). Eight normal gingival samples were collected from dogs with no history and clinical signs of oral cavity or cancerous problems (age range 8 months-13 years). Affected dogs were measured for tumor size (T), lymph node examined (N) and thoracic and abdominal radiographed (M). Clinical staging of the affected dogs was done according to TNM staging. The samples were immediately collected after anesthesia and from freshly necropsied dogs submitted to the Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University (Thailand). Samples were obtained with the consent of owners following the ethical guidelines required under the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand (Protocol No. 1531005).

Isolation of samples

Tissues were bisected for histopathological and IHC study and for quantitative reverse transcription polymerase chain reaction (qRT-PCR) study. For the histopathological and IHC study, samples were immersion fixed in 10% neutral buffered formalin for 24 h, followed by standard tissue processing and paraffin

embedding. For quantitative reverse transcriptase polymerase chain reaction, samples were kept in RNALater solution overnight at 4 °C and stored at -20 °C until being processed.

Histopathology and immunohistochemistry

Tumor samples in paraffin-embedded blocks were cut into sections (4 μm thickness) and mounted on glass slides. Slides were deparaffinized in xylene, rehydrated in graded ethanol, and then stained with hematoxylin and eosin (H&E) for routine histopathologic evaluation. IHC was used to confirm the diagnosis of amelanotic melanoma with a mouse monoclonal antibody against human Melan-A (Dako, Glostrup, Denmark). Antigen retrieval was performed by microwave oven in 0.01 M sodium citrate, pH 6.0, at 800 W for 10 min. Slides were incubated with anti-Melan-A antibody at the dilution 1:50 at 4 °C for 12 h. For Ki-67, antigen retrieval was performed by autoclave at 121 °C for 20 min in 0.01 M citrate buffer, pH 6.0. For E-cadherin and syndecan 1, antigens retrieval was performed by microwave oven in 0.01 M sodium citrate, pH 6.0, at 800 W for 10 min. Endogenous peroxidase was quenched by incubating the slides in 3% hydrogen peroxide in methanol for 10 min. Nonspecific immunoglobulin binding was blocked with 1-3% bovine serum albumin at 37 °C for 20 min (Merck, Rockland, MA). A mouse monoclonal Ki-67 antibody (MIB-

1) (Dako, Glostrup, Denmark) at the dilution 1:50 was used for investigation of the cell proliferation. To assess the localization of the E-cadherin and syndecan 1 proteins, a mouse against canine E-cadherin antibody (BD Biosciences, Franklin Lakes, NJ) at the dilution 1:100 and a rabbit against human syndecan 1 antibody (Abcam, Cambridge, UK) at the dilution 1:200, respectively, were used. All antibodies were incubated at 4 °C overnight.

Primary antibody binding was detected by a polymer-based non avidin-biotin system, EnVision detection system (Dako, Glostrup, Denmark), incubating at 37 °C for 45 minutes, and visualized with a 3,3´-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Abcam, Cambridge, MA). Slides were counterstained with Mayer's hematoxylin. For Melan-A Ab, a positive control was a canine oral melanotic melanoma section. For other Abs, a positive control was feline mammary gland carcinoma section and a negative control slide was a freshly necropsied canine gingival section, prepared with and without the primary antibody, respectively. The staining results were evaluated to positively or negatively cytoplasmic area except Ki-67 staining which was evaluated to positively or negatively nuclear area.

RNA isolation

Total RNA was extracted from OM, OSCC and normal gingival tissues, using Nucleospin RNA kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's protocol. The total RNA samples were treated twice with TURBO DNase (Thermo Fisher Scientific/Life Technologies, Grand Island, NY) for 30 min at 37 °C each round to remove contaminating genomic DNA and pseudogenes. The concentration of the RNA was determined using a NanoDrop ND-1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Waltham, MA) by measuring the absorbance at a wavelength of 260 nm (A_{260}) whereas the A_{260}nm/A_{280} nm ratio reflects the RNA purity, ranged from 1.8 to 2.2. The integrity of the RNA was determined by 1% agarose gel electrophoresis to assess the 28S and 18S bands.

Primer design and testing

Primer sequences of *RPS5* and *RPS19* have been previously described (Schlotter et al 2009). Other primers were developed using Primer 3 version 0.4.0 software (http://gmdd.shgmo.org/primer3/?seqid=47) (Rozen and Skaletzky 1999). Specificity of each primer was verified using the In-Silico PCR program (http://genome.ucsc.edu/), a virtual PCR against the canine reference genome (CanFam 3.1, September 2011 assembly), and the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast), returning Genbank accession numbers. DNA

sequencing was performed to verify the genes. The primer sequences, accession numbers, and amplicons are depicted in Table 1.

Table 1 Primers used in the present study

Gene	Accession number	Primers (5’-->3’)	Amplicon (bp)	Tm* (°C)
Beta-actin (ACTB)	AF_021873.2	Fwd 5’-atggaatcatgcggtatccac-3’	141	60.38
		Rev 5’-cttctgcatcctgtcagcaa-3’		58.54
Beta-2 microglobulin (B2M)	XM_003640047.2	Fwd 5’-tcccccaaagattcaagtgt-3’	85	57.86
		Rev 5’-atggaaccctgacacgtagc-3’		58.46
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	NM_001003142.1	Fwd 5’-gccctcaatgaccactttgt-3’	101	58.43
		Rev 5’-tccttggaggccatgtagac-3’		58.53
Ribosomal protein L13a (RPL13a)	XM_003432726.2	Fwd 5’-atcccaccaccctatgacaa-3’	152	58.50
		Rev 5’-tcctccagggttgctgttac-3’		58.58
Ribosomal protein S5 (RPS5)	XM_533568.4	Fwd 5’-tcactggtgagaaccccct-3’	141	58.89
		Rev 5’-cctgattcacacggcgtag-3’		58.68
Ribosomal protein S19 (RPS19)	XM_003639381.2	Fwd 5’-ccttcctcaaaaagtctggg-3’	95	57.28
		Rev 5’-gttctcatcgtagggagcaag-3’		57.46
E-cadherin (CDH1)	XM_536807.3	Fwd 5’-ggtgctcacatttcccagtt-3’	100	58.43
		Rev 5’-aaatgggcctttctcgtttt-3’		58.54
Syndecan 1 (SDC1)	XM_540099.4	Fwd 5’-ccaccatcagatctcggttc-3’	117	58.89
		Rev 5’-tgagtggagactccgtcctc-3’		58.77
Matrix metallo-proteinase-2 (MMP2)	AF_147750.1	Fwd 5’-ctggctgtgcaatacctgaa-3’	143	58.31
		Rev 5’-gtttcgatggtgctctggtc-3’		59.09
Matrix metallo-proteinase-7 (MMP7)	NM_001242726.1	Fwd 5’-cactggattcggtgtcattg-3’	126	58.35
		Rev 5’-agcttctctttgggacagca-3’		58.60
Matrix metallo-proteinase-9 (MMP9)	NM_001003219.1	Fwd 5’-catgacatcttccagtaccaag-3’	115	55.70
		Rev 5’-caaaggtcacgtagcccact-3’		58.65
Matrix metallo-proteinase-14	AY_534615.1	Fwd 5’-cctacttcttccggggaaac-3’	123	58.49
		Rev 5’-tgaatgaccctctgggagac-3’		58.45

(MMP14)				
tissue inhibitor matrix metallo-proteinase-1 (TIMP1)	AB_016817.1	Fwd 5'-ctcaccagagaacccaccat-3' Rev 5'-cctgatgacgatttgggagt-3'	147	58.37 58.38
tissue inhibitor matrix metallo-proteinase-2 (TIMP2)	NM_001003082.1	Fwd 5'-agcagcacccagaagaagag-3' Rev 5'-gtccatccagaggcactcat-3'	120	58.21 58.48

*Tm calculator by http://www6.appliedbiosystems.com/support/techtools/calc/

Quantitative reverse transcription PCR

DNase-treated RNA was converted to cDNA using the SuperScript III First strand synthesis system for RT-PCR (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, one microgram of RNA was reverse transcribed in a 20 µL reaction containing 50 ng random primers, 40 U RNase inhibitor and 200 U Superscript III enzyme. qRT-PCR was performed by SYBR Green chemistry (KAPA SYBR Fast qPCR Master Mix Universal; KAPA Biosystems, Cambridge, MA) and analyzed on the Rotor Gene 3000 Thermal Cycler (Qiagen, Hilden, Germany). PCR reactions were performed as previously described (Theerawatanasirikul et al 2012). Primers were used at 200 nM each and cDNA at 25 ng in 10-µL reactions. Thermal cycling conditions were performed according to the manufacturer's instructions: 95 °C for 2 min followed by 40 cycles at 95 °C for 3 s, 60 °C for 20 s and 72 °C for 1 s. Each

reaction was performed in duplicate in three independent runs. Only Cq from samples with the duplicate Cq difference < 1 were further analysis. A melting curve from 72 °C with a rate of 1 °C per second up to 95 °C was analyzed to verify the purity of the PCR products. The real-time data analysis was performed by REST-2009 (Relative Expression Software Tool) software (Pfaffl et al 2002) with a detection threshold at 0.1.

Statistical Analysis

Statistical analysis of immunohistochemical staining data was conducted using a GraphPad Prism software, version 5.0 (San Diego, California). For protein staining scores, statistical differences were performed by non-parametric Kruskal-Wallis test and Dunn's multiple comparison post test. Results with a P value <0.05 were considered significant.

For the reference gene selection, five algorithms were used to rank the reference genes according to their expression stability, including geNorm, Normfinder, BestKeeper, comparative dCt, and RefFinder (Vandesompele et al 2002; Andersen et al 2004; Pfaffl et al 2004; Silver et al 2006; Taki et al 2014). The concordance from at least three algorithms was used to determine the most stably expressed reference gene to normalize target genes in OM and OSCC (Jacob et al 2013). The integrity of

each sample was determined by intrinsic variance (InVar) of expression values, using BestKeeper software. Strong deviating samples due to any technical errors such as sample degradation and incomplete reverse transcription were discarded. For the real-time PCR results, REST 2009 software with a pair-wise fixed reallocation randomization test was used to show significant differences of target gene expression between normal and cancerous tissues and also between early and late stage OM. Results with a P value <0.05 were considered significant.

4. Results

Histopathology

Histopathology of melanotic melanoma, amelanotic melanoma, and squamous cell carcinoma is shown in Fig. 1. Melan-A immunohistochemical staining was performed to confirm amelanotic melanoma diagnosis. Melanoma cases were histopathological diagnoses based on cell types; melanotic or amelanotic, epithelioid, spindle or mixed cell types. The OSCC samples were histopathological classified as 5 cases of well and 2 cases of poorly differentiated. The OM samples, either melanotic and amelanotic, were categorized according to the WHO staging scheme and cell morphology pattern (Table 2).

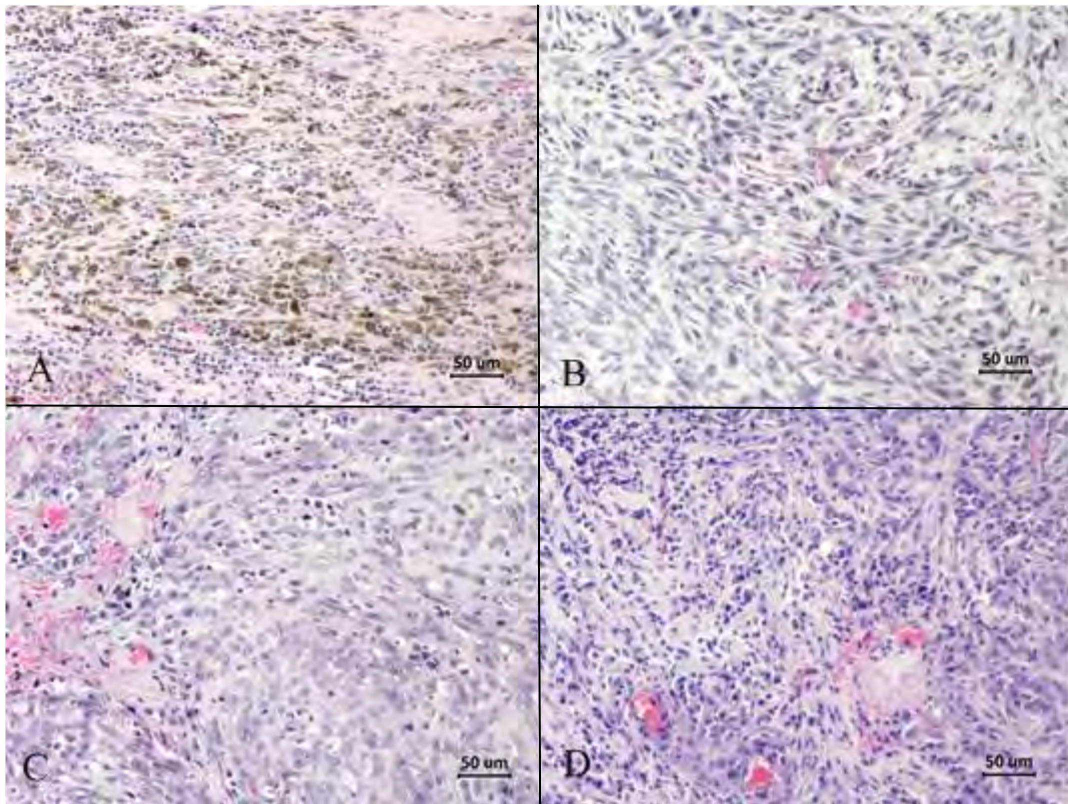

Fig. 1 Histopathology of melanotic melanoma (A), amelanotic melanoma (B), well-differentiated squamous cell carcinoma (C), poor-differentiated squamous cell carcinoma (D) (scale bar = 50 µm)

Table 2 Tumor, node, metastasis (TNM) and histopathological classifications of 12 dogs with oral malignant melanoma

Dog number	TNM Stage	Melanotic (M)/ amelanotic (A)	Cell morphology
1	1	M	Epithelioid
2	2	A	Epithelioid
3	2	A	Spindle
4	3	M	Epithelioid
5	3	M	Epithelioid
6	3	M	Epithelioid
7	3	A	Epithelioid
8	3	A	Epithelioid
9	3	A	Epithelioid
10	3	A	Epithelioid
11	3	A	Epithelioid
12	4	M	Epithelioid

Selection of reference genes and qRT-PCR of CDH1 SDC1 MMP2, MMP 7, MMP 9, MMP 14, TIMP1, TIMP2

Based on the integrity of cDNA samples determined by the BestKeeper algorithm, two strong deviating samples of amelanotic melanoma stage 3 with high InVar values, 10.84 and 130.7, were excluded to increase consistence and reliability of the data analysis. For the analysis of suitable reference genes in canine OM and/or OSCC, a cohort of top three genes from most algorithms for OM and/or OSCC were *ACTB*, *RPS5* and *RPS19* (Tables 3-5). A similar combination was obtained when we used these algorithms calculated by the web-based program despite different values

(Table 6). However, if two samples with high InVar values were included, reference genes of the top rank in the OM plus OSCC and OM alone analyzed by geNorm either by the original program itself or by the web-based program were *RPS5/RPS19* and *RPL13a*.

Table 3 Ranking of the reference genes in canine oral melanoma (*n* = 12) and oral squamous cell carcinoma (*n* = 7) from high to low stability by various algorithms. Stability values are in brackets.

Rank	geNorm	NormFinder	BestKeeper	dCt*	RefFinder
1	RPS5/RPS19 (0.696)	ACTB (0.002)	RPS19 (1.050)	ACTB (1.112)	RPS19 (1.414)
2	-	RPS19/RPS5 (0.011)	RPS5 (1.099)	RPS519 (1.196)	ACTB (1.732)
3	ACTB (0.904)	-	ACTB (1.149)	RPS5 (1.214)	RPS5 (2.06)
4	RPL13a (1.005)	RPL13a (0.018)	RPL13a (1.164)	RPL13a (1.348)	RPL13a (4.00)
5	GAPDH (1.163)	GAPDH (0.019)	GAPDH (1.193)	GAPDH (1.518)	GAPDH (5.00)
6	B2M (1.335)	B2M (0.024)	B2M (1.208)	B2M (1.673)	B2M (6.00)

*dCt - Comparative delta threshold cycle

Table 4 Ranking of the reference genes in canine oral melanoma (*n* = 12) from high to low stability by various algorithms. Stability values are in brackets.

Rank	geNorm	NormFinder	BestKeeper	dCt	RefFinder
1	RPS5/RPS19 (0.642)	ACTB (0.005)	RPS19 (0.866)	ACTB (1.010)	RPS19 (1.732)
2	-	RPS5 (0.006)	GAPDH (0.896)	RPS5 (1.016)	RPS5 (1.861)
3	ACTB (0.728)	RPS19 (0.008)	RPS5 (0.903)	RPS19 (1.049)	ACTB (1.861)
4	RPL13a (0.915)	GAPDH (0.016)	ACTB (0.992)	RPL13a (1.292)	GAPDH (3.976)
5	GAPDH (1.021)	RPL13a (0.018)	RPL13a (1.021)	GAPDH (1.345)	RPL13a (4.229)
6	B2M (1.223)	B2M (0.025)	B2M (1.217)	B2M (1.626)	B2M (6.000)

Table 5 Ranking of the reference genes in canine oral squamous cell carcinoma (*n* = 7) from high to low stability by various algorithms. Stability values are in brackets.

Rank	geNorm	NormFinder	BestKeeper	dCt	RefFinder
1	RPS5/RPS19 (0.829)	ACTB (0.003)	RPS5 (0.970)	ACTB (1.136)	RPS19 (1.68)
2	-	RPS19 (0.012)	RPS19 (1.027)	RPS19 (1.256)	ACTB/RPS5 (1.732)
3	ACTB (1.020)	RPS5 (0.013)	ACTB (1.174)	RPS5 (1.296)	-

4	RPL13a (1.116)	GAPDH (0.016)	RPL13a (1.179)	GAPDH (1.401)	RPL13a (4.472)
5	GAPDH (1.240)	B2M (0.020)	B2M (1.198)	RPL13a (1.468)	GAPDH (4.681)
6	B2M (1.340)	RPL13a (0.021)	GAPDH (1.379)	B2M (1.548)	B2M (5.733)

Table 6 Ranking of the reference genes in canine oral melanoma (*n* = 12) and/or oral squamous cell carcinoma (*n* = 7) from high to low stability with geNorm and NormFinder programs, calculated by the web-based program (http://www.leonxie.com/referencegene.php). Stability values are in brackets.

Rank	OM+OSCC		OM		OSCC	
	geNorm	NormFinder	geNorm	NormFinder	geNorm	NormFinder
1	RPS5/RPS19 (0.697)	ACTB (0.355)	RPS5/RPS19 (0.642)	ACTB (0.353)	RPS5/RPS19 (0.818)	ACTB (0.460)
2	-	RPS19 (0.694)	-	RPS5 (0.440)	-	RPS19 (0.786)
3	ACTB (0.905)	RPS5 (0.772)	ACTB (0.728)	RPS19 (0.505)	ACTB (1.034)	RPS5 (0.911)
4	RPL13a (1.016)	RPL13a (0.968)	RPL13a (0.915)	RPL13a (0.985)	RPL13a (1.140)	GAPDH (1.063)
5	GAPDH (1.179)	GAPDH (1.241)	GAPDH (1.021)	GAPDH (1.091)	GAPDH (1.252)	RPL13a (1.167)
6	B2M (1.343)	B2M (1.451)	B2M (1.223)	B2M (1.469)	B2M (1.351)	B2M (1.283)

The geNorm analysis suggested that the top three genes, *ACTB*, *RPS5* and *RPS19*, had high expression stability with M values lower than the cut off value at 1.5 (Vandesompele et al 2002). V2/3 - V5/6 scores of all OM and/or OSCC ranged from 0.209-0.345. Since the V scores were higher than 0.15, we used each reference gene and also the combination of all and the three best reference genes (*ACTB*, *RPS5* and *RPS19*) for further normalization to the target gene expression as recommended by the geNorm manual (medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf). The REST 2009 software was used to analyze target gene expression in canine normal oral cavity, OM and/or OSCC, irrespective of TNM stage and histological grade (Pfaffl et al 2002). CDH1 and SDC1 are cell adhesion molecules and also tumor suppressor proteins. As expected, lower expression of *CDH1* and *SDC1* was observed in the OM and/or OSCC compared to the normal tissues (Table 7). From the pair-wise correlation analyses, we found that *ACTB*, *RPS5*, *RPS19* and *RPL13a* in the OM and OSCC samples showed strong correlation with the BestKeeper Index ($P < 0.001$) with high coefficient of correlation [$0.669 < r < 0.935$], whereas the target genes, *CDH1*, *SDC1*, did not exhibit any significant correlation with the Index with $r = 0.331$ ($P = 0.106$) and $r = 0.093$ ($P = 0.657$), respectively.

Table 7 Expression ratios of *CDH1* (upper row) and *SDC1* (lower row) against several reference genes in the oral melanoma and/or oral squamous cell carcinoma

	OM+OSCC		OM		OSCC	
	Expression ratios	*P* values	Expression ratios	*P* values	Expression ratios	*P* values
All reference genes	0.108**	0.002	0.174**	0.003	0.056**	0.008
	0.074**	0.001	0.047**	0.001	0.146**	0.002
RPS5/RPS19/ACTB	0.104**	0.005	0.156**	0.004	0.061*	0.016
	0.078**	0.001	0.042**	0.001	0.159**	0.003
ACTB	0.072**	0.002	0.127**	0.001	0.035**	0.002
	0.049***	0.0001	0.034***	0.0001	0.093**	0.001
RPS5	0.144*	0.011	0.193*	0.017	0.094*	0.047
	0.099**	0.002	0.052**	0.002	0.246*	0.023
RPS19	0.110**	0.007	0.154**	0.006	0.068*	0.023
	0.075***	0.0001	0.041**	0.002	0.177**	0.004
RPL13a	0.188*	0.026	0.249**	0.009	0.126	0.054
	0.129**	0.003	0.067**	0.001	0.327	0.099
B2M	0.054**	0.001	0.091**	0.006	0.026**	0.003
	0.037***	0.0001	0.024***	0.0001	0.067**	0.005
GAPDH	0.141*	0.010	0.326*	0.037	0.043**	0.009
	0.097**	0.001	0.088**	0.001	0.111**	0.004

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

The expression of *MMP2* and *MMP14* was shown to be upregulated at all stages of the OM whereas the expression of *MMP7* gene was downregulated. However, in stages 1-2 and stages 3-4, *TIMP1* and *TIMP2* expression was also increased, respectively (Table 8).

Table 8 Expression ratios of *CDH1, SDC1, MMP2*, *MMP7*, *MMP9*, *MMP14*, *TIMP1* and *TIMP2* against 3 reference genes, *ACTB*, *RPS5* and *RPS19*, in the oral melanoma at different stages.

Genes	Stages 1-2	Stages 3-4	Stages 1-4
CDH1	0.112*	0.195*	0.156*
SDC1	0.034*	0.048*	0.042*
MMP2	233.779**	253.261**	245.280**
MMP7	0.042*	0.093*	0.068*
MMP9	0.441	0.543	0.500
MMP14	6.185**	5.105**	5.513**
TIMP1	17.240**	2.045	4.798
TIMP2	1.949	4.762**	3.331

*Gene downregulation with $P < 0.05$
**Gene upregulation with $P < 0.05$

Immunohistochemical expression of Ki-67, CDH1 and SDC1 in canine OM

IHC for Ki-67 antigen was confined to nuclei of nucleated cancer cells, indicating hyperplastic proliferation rate, and it could be distinguished from granular brown cytoplasmic melanin pigment in the melanotic melanoma (Fig 2, Table 9). One of the normal sample was hyperplasia, hence, the Ki-67 standard deviation (SD) was high. However, the scores of cancer cells, in general, were higher than normal. For CDH1 and SDC1 protein expression, the percent positive areas were decreased in cancer cells compared to a normal control (Figs 3 and 4, Table 9).

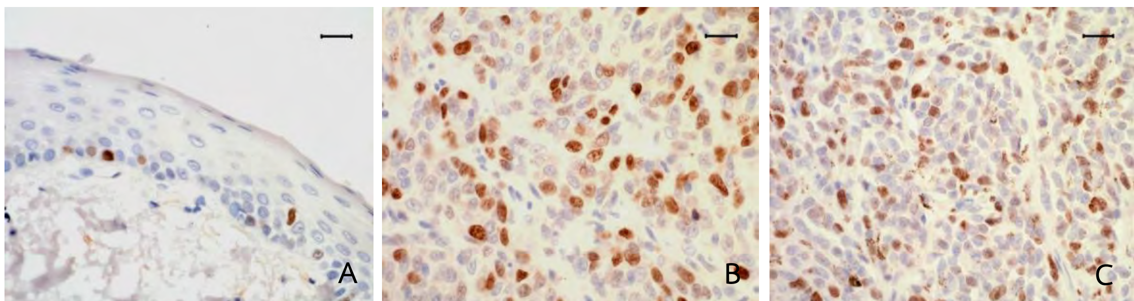

Fig. 2 Immunohistochemical staining for Ki-67 in canine oral melanoma. Ki-67 expression in normal canine gingiva (A), amelanotic melanoma (B) and melanotic melanoma (C). Scale bar = 10 µm.

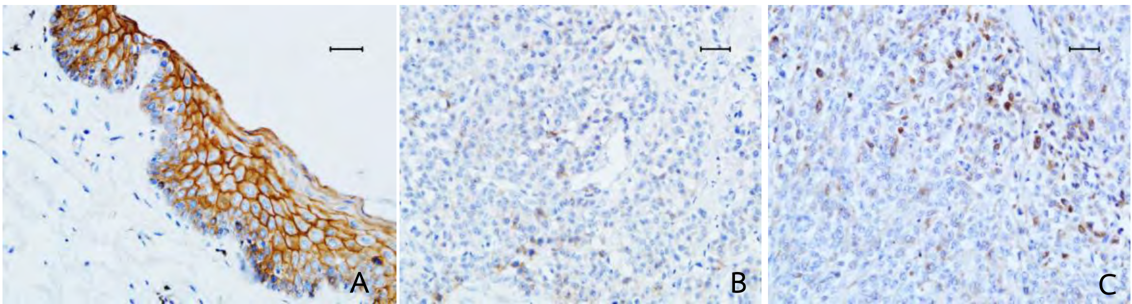

Fig. 3 Immunohistochemical staining for E-cadherin in canine oral melanoma. E-cadherin expression in normal canine gingiva (A), amelanotic melanoma (B) and melanotic melanoma (C). Scale bar = 20 µm.

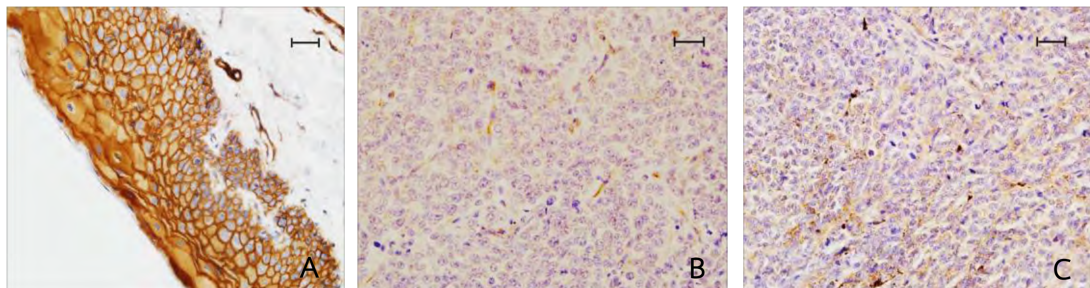

Fig. 4 Immunohistochemical staining for syndecan1 in canine oral melanoma. Syndecan1 expression in normal canine gingiva (A), amelanotic melanoma (B) and melanotic melanoma (C). Scale bar = 20 µm.

Table 9 Immunohistochemical scores for Ki-67 (as percent positive nuclei), and for E-cadherin and syndecan1 (as percent positive area) in canine oral melanoma at various TNM stages

	Normal (Mean ± SD)	Stages 1-2 (Mean ± SD)	Stages 3-4 (Mean ± SD)	Stages 1-4 (Mean ± SD)
Ki-67	6.61 ± 6.31	48.48 ± 7.35*	27.24 ± 18.01	34.32 ± 18.18**
CDH1 (E-cadherin)	59.34 ± 9.92	17.84 ± 6.42*	20.79 ± 11.36**	19.81 ± 9.77**
SDC1 (Syndecan 1)	59.30 ± 13.10	18.89 ± 10.20*	20.85 ± 12.70**	20.20 ± 11.49**

*Significantly different from a normal group at $P < 0.05$, using Kruskal-Wallis test and Dunn's multiple comparison post test

**Significantly different from a normal group at $P < 0.01$, using Kruskal-Wallis test and Dunn's multiple comparison post test

5. Discussion

In this study, the suitable reference genes for canine oral cancers, OM and non-tonsillar OSCC, were suggested, using various statistical algorithms. Excluding high InVar values, most algorithms represented a cohort of *RPS5*, *RPS19* and *ACTB* as the top three reference genes. BestKeeper represented similar top-ranking genes to the others merely in the combination of OM and OSCC samples. The divergent ranks of the top three genes calculated by different normalization approaches revealed the

necessity to use more than one algorithm to analyze results. The web-based program gave similar rank of candidate reference genes as the original programs and we found this tool feature useful in practice. The RefFinder was also used in selection of reference genes in chicken tissues (Bagés et al 2015). Since the top three reference genes from the geNorm analysis were changed to be a cohort of *RPS5/RPS19* and *RPL13a* when samples with high InVar values were included, it is important to verify the sample's integrity before selection of normalization methods. Although *CDH1* and *SDC1* were shown to be down-regulated when normalized against most reference genes in the OM and/or OSCC, irrespective of tumor stage and histopathological diagnosis, the high *P* value of *CDH1* expression ratios against *RPS5* (*P* = 0.047) (Table 7) in OSCC supported the concept of utilization of more than one reference gene to normalize target gene. *CDH1* gene encodes a tumor suppressor protein and decreased CDH1 protein expression was demonstrated by IHC in this study, corresponding to the previous results in canine melanotic tumors and human melanocytic tumor cell lines (Poser et al 2001; Han et al 2013). Similar to CDH1 results, SDC1 was also shown to be downregulated in this work. Loss of SDC1 protein in melanoma cells was reported to be involved in tumor cell invasion and metastasis (Reiland et al 2004). Coordinated decrease of CDH1 and SDC1 was reported in

malignant mammary tumor cells (Leppä et al 1996). The *CDH1* and *SDC1* genes showed no correlation with the BestKeeper Index of the candidate reference genes. Since both *RPS5* and *RPS19* encoded proteins with related functions and both showed strong correlation with the BestKeeper Index, one of them might be selected together with *ACTB* for the normalization of OM and OSCC samples.

The selected reference genes were used to normalize several target genes of OM. In the present study, the gene expression of *MMP2* and *MMP14* was found to be upregulated whereas *CDH1*, *SDC1* and *MMP7* expression was downregulated. From IHC results, Ki-67 expression was increased in tumor cells whereas CDH1 and SDC1 expression was decreased. Ki-67 protein is a cellular marker for proliferation (Schlüter et al 1993). It can be exclusively detected within the cell nucleus of the cell during interphase in cell cycle. In this study, the expression of Ki-67 was increased in OM tissues. In canine melanoma, Ki-67 was also used as one of prognostic markers of the diseases (Roels et al 1999; Millanta et al 2002). Ki-67 was also used as a cell proliferation marker in other cancers, including brain and breast cancers (Ide et al 2011; Santos et al 2013). Upregulation of *MMP2* and *MMP14* in all stages of the OM disease showed the strong correlation with the disease. High expression of MMP14 and low levels of TIMP2 were required to activate proMMP2, indicating cancer

progression. On the other hand, TIMP2 at high concentrations can inhibit unbound active MMP2 (Goldberg et al 1992; Gomez et al 1997; Bernardo and Fridman 2003; Toth et al 2003). In this study upregulation of *TIMP2* were observed in the late stage of the disease for unknown reason, probably an attempt to inhibit overexpression of MMP2. On a side note, *TIMP1* was upregulated merely at the early stages of the disease where lymph node invasion and metastasis were not provoked, probably indicating an invasion and metastasis suppressor. Overexpression of TIMP1 in melanoma cells had been previously demonstrated to reduce metastasis (Khokha 1994).

6. Conclusion

This study validated the combination of *ACTB*, *RPS5* and *RPS19* as the best reference genes for qRT-PCR analysis of canine OM and OSCC tissues. The combination of reference genes and the utilization of more than one algorithm are recommended. This study presented the downregulation of *CDH1* and *SDC1* genes in canine OM and non-tonsillar OSCC. In addition, it also showed the alteration of MMP family gene expression in canine OM.

7. Suggestion for Future Work

For the future work, since lower expression of tumor suppressing genes and the upregulation of *MMP2* and *MMP14* showed the association with the OM, regardless of the disease stages whereas upregulation of *TIMP1* and *TIMP2* was observed in the early and late stages of the OM, respectively. These gene expression profiles should be further studied for the possibility to be used as diagnostic and/or prognostic markers together with drug targets of the OM.

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ตารางแสดงรายละเอียดการใช้เงิน
รายงานฉบับสมบูรณ์
ระหว่าง 1 กันยายน 2556 ถึง 31 สิงหาคม 2557
หมวดวิทยาศาสตร์ชีวภาพ

ลำดับที่	เลขที่ใบเสร็จ/ใบสำคัญรับเงิน	ว.ป.ด.	รายการ	จำนวนเงิน (บาท)
ทุนงวดแรก 214,500 บาท				
1.	RV 36773	16.09.56	Oligosynthesis 0.025 μmol	1,198.40
2.	RV 43953	16.09.56	Oligosynthesis 0.025 μmol	1,797.60
3.	RV 43956	18.09.56	Oligosynthesis 0.025 μmol	6,306.58
4.	5406638	27.09.56	DNA sequencing	5,033.28
5.	43955	27.09.56	FITC-100 Modification FITC TET-100 Modification TET	24,342.50
6.	101017	27.09.56	100 bp DNA ladder	6,355.80
7.	RV 45790	10.10.56	Oligosynthesis 0.025 μmol	4,194.40
8.	RC-5601258	10.10.56	dNTPs	3,595.20
9.	RC-5601260	10.10.56	RNALater	4,280.00
10.	RC-5601257	18.10.56	RNALater stabilization reagent	4,354.90
11.	57485	18.10.56	Liquid nitrogen	2,140.00
12.	RV 36771	18.10.56	Oligosynthesis 0.025 μmol	2,396.80
13.	RV 36775	18.10.56	DNA sequencing	2,140.00
14.	RV 36774	21.10.56	Oligosynthesis 0.025 μmol	1,797.60
15.	59448	21.10.56	HiYield Gel/PCR DNA Mini kit	2,514.50
16.	62849	21.10.56	Microcentrifuge tube opener	1,123.50
17.	68592	28.10.56	Wireless presenter	1,190.00
18.	RV 46600	28.10.56	Oligosynthesis 0.025 μmol	1,198.40
19.	RV 46601	28.10.56	DNA Sequencing	3,852.00
20.	RV 46602	28.10.56	Agarose powder low EEO	2,996.00
21.	101672	28.10.56	Taq DNA Polymerase	7,490.00
22.	L56-09419	28.10.56	FTA Elute micro card	4,815.00
23.	RC-5601768	28.10.56	Anti-syndecan1 antibody 1.5 mL microtubes 0.5 mL microtubes	18,142.49
24.	59360	28.10.56	Liquid nitrogen	2,140.00

25.	32636	28.10.56	SSIII 1st strand synthesis	17,655.00
26.	32635	01.11.56	RNALater	9,095.00
27.	102681	01.11.56	Agarose	3,210.00
28.	32634	04.11.56	10 μL micro tip, extra long	2,514.50
29.	R56-01483	04.11.56	Positive charged slides	1,440.00
30.	47727	04.11.56	Agarose	10,111.50
31.	RC-5602344	04.11.56	dNTP mix	5,392.80
32.	RC-5602345	04.11.56	SYBR Fast universal master mix	17,120.00
33.	700/10075623	18.11.56	Bis-acrylamide Isopropanol Non fat powered milk DNaseI	11,556.00
34.	ค่าจ้างผู้ช่วยวิจัย	09.56- 02.57		21,000.00
35.	ดอกเบี้ย	23.12.56		245.37
ทุนงวดที่ 2 143,000 บาท				
36.	RC-5701517	07.03.57	SYBR Fast universal master mix	17,436.72
37.	RC-5700117	12.03.57	1.5mL microtubes 200μL pipette tips EtBr destroyer spray GEL/PCR purification mini kit	12,849.20
38.	36224	14.03.57	SSIII Reverse Transcriptase	19,260.00
39.	38373	21.03.57	10,200,1250μL pipette tips PCR tubes Storage box 100 holes	7,621.70
40.	35247	04.04.57	Filter tip 10 uL	11,770.00
41.	39874	25.04.57	TURBO DNase	14,766.00
42.	39875	08.05.57	SSIII 1st Strand Synthesis System	22,149.00
43.	5707055	22.05.57	T-PER reagent HALT Protease inhibitor cocktail	16,473.51
44.	ค่าจ้างผู้ช่วยวิจัย	03.57- 08.57		21,000.00
45.	ดอกเบี้ย	30.06.57		117.89
46.	ดอกเบี้ย	07.08.57		186.71

รวม 358,365.85 บาท (สามแสนห้าหมื่นแปดพันสามร้อยหกสิบห้าบาทแปดสิบห้าสตางค์)