รายงานวิจัยฉบับสมบูรณ์

Project: Study of immunohistochemical expression of sphingosine kinase, sphingosine-1 phosphate receptor-1, mast cells; relationship to clinical variables in canine mammary gland tumor

โครงการ: การศึกษาการแสดงออกทางอิมมูโนฮีสโตเคมีของสฟิงโกซีนไคเนส-1 ตัวรับชนิดสฟิงโกซีน-1-ฟอสเฟต และมาสต์เซลล์ ที่มีความสัมพันธ์ต่อค่าทางคลินิกในเนื้องอกเต้านมของสุนัข

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Abstract

Project Code: TRG5780225

Project Title: Study of immunohistochemical expression of sphingosine kinase, sphingosine-1 phosphate receptor-1, mast cells; relationship to clinical variables in canine mammary gland tumor.

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Project Period: 7 years (inclusive of the approved extended period)

The present study was to evaluate the expression of sphingosine kinase1 (SPHK1), sphingosine-1 phosphate receptor-1 (S1PR-1), mast cell density (MCD) and microvessel density (MVD) in association with canine mammary gland tumor. Tumor tissues from mastectomy were classified into simple adenoma and simple carcinoma, tubular type based on histopathological features. The evaluation was made using Hematoxylin and Eosin (H&E stain), Toluidine blue stain and Immunohistochemistry on paraffin-embedded tissues. The result showed significant upregulation of SPHK1 expression in cytoplasm of neoplastic cells, increased MCD and MVD density in simple carcinoma, tubular type as compared to tissues of normal mammary tissue and simple adenoma ($p < 0.05$). Positive correlation between SPHK1 expression and MVD, MCD and MVD and also between SPHK1 expression and metastasis was documented in simple carcinoma, where there were no other correlation of other variables in normal control, simple adenoma and simple carcinoma. This study suggests that overexpression of SPHK1, increased MCD and increased MVD are features in mammary carcinoma by which the finding of overexpression of SPHK1 might partly be a predictive marker for metastasis of this disease.

Keywords canine mammary gland tumor, mast cell, sphingosine kinase-1, sphingosine-1 phosphate receptor-1, angiogenesis

บทคัดย่อ

รหัสโครงการ: TRG5780225

ชื่อโครงการ: การศึกษาการแสดงออกทางอิมมูโนฮีสโตเคมีของสฟิงโกซีนไคเนส-1 ตัวรับชนิดสฟิงโกซีน-1-ฟอสเฟต และมาสต์เซลล์ (mast cell) ที่มีความสัมพันธ์ต่อค่าทางคลินิกในเนื้องอกเต้านมของสุนัข

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ระยะเวลาโครงการ: 7 ปี

การศึกษานี้เพื่อประเมินการแสดงออกของสฟิงโกซีนไคเนส-1 (sphingosine kinase1, SPHK1) ตัวรับชนิดสฟิงโกซีน-1-ฟอสเฟต (sphingosine-1 phosphate receptor-1, S1PR-1) ความหนาแน่นมาสต์เซลล์ (mast cell density, MCD) และความหนาแน่นของหลอดเลือดขนาดเล็ก(microvessel density, MVD) ซึ่งมีความสัมพันธ์ในเนื้องอกเต้านมสุนัข จากตัวอย่างการผ่าตัดซึ่งถูกจำแนกเป็น Simple adenoma และ Simple carcinoma ชนิด tubular type ตามลักษณะทางจุลพยาธิวิทยา และประเมินโดยการย้อมสีฮีมาทอกซิลินและอีโอซิน สีโทลูอิดีนบลู และอิมมูโนฮีสโตเคมี บนชิ้นเนื้อในพาราฟิน ผลการทดลองพบการแสดงออกของสฟิงโกซีนไคเนส-1 ในเซลล์มะเร็งสูงขึ้นอย่างมีนัยสำคัญทางสถิติ ร่วมกับความหนาแน่นของมาสต์เซลล์และหลอดเลือดในตัวอย่างมะเร็งชนิด simple carcinoma ชนิด tubular type เมื่อเปรียบเทียบกับกลุ่มเนื้อเยื่อเต้านมปกติ และ กลุ่ม simple adenoma ($p < 0.05$) และพบความสัมพันธ์เชิงบวกระหว่าง การแสดงออกของสฟิงโกซีนไคเนส-1 ต่อความหนาแน่นของหลอดเลือด ความสัมพันธ์เชิงบวกระหว่างความหนาแน่นของมาสต์เซลล์ต่อความหนาแน่นของหลอดเลือด และการแสดงออกของสฟิงโกซีนไคเนส-1 ต่อการแพร่กระจายของเซลล์มะเร็ง ในกลุ่มเนื้องอก simple carcinoma และไม่พบความสัมพันธ์ระหว่างตัวแปรอื่นในกลุ่มควบคุม กลุ่ม simple adenoma และกลุ่ม simple carcinoma การศึกษานี้แสดงให้เห็นถึง ระดับการแสดงออกของ สฟิงโกซีนไคเนส-1 ที่สูงขึ้นและความหนาแน่นของมาสต์เซลล์และหลอดเลือดที่เพิ่มขึ้นเป็นลักษณะของเนื้องอกเต้านมชนิดร้ายแรงและค่าดังกล่าวอาจใช้เป็นตัวบ่งชี้การแพร่กระจายของเซลล์มะเร็งของโรคนี้

คำหลัก: เนื้องอกเต้านมสุนัข มาสต์เซลล์ สฟิงโกซีนไคเนส-1 ตัวรับชนิดสฟิงโกซีน-1-ฟอสเฟต การสร้างหลอดเลือดใหม่

Executive summary

Project Title: **Study of immunohistochemical expression of sphingosine kinase, sphingosine-1 phosphate receptor-1, mast cells; relationship to clinical variables in canine mammary gland tumor**

Investigator: Dr. Panop Wilainam
Faculty of Veterinary Science, Mahidol University

1. Background and Rationale

Mammary gland tumors have been regarded as the second most commonly diagnosed tumor in female dogs of which they have been documented for being a major cause of death. Malignant tumors are fast growing, highly invasive into surrounding tissue, high recurrence after surgical removal and have highly metastatic potential of neoplastic cells to distant organs.

Angiogenesis or neovascularization is a complex process from which the formation of new blood vessels from the preexisting vasculatures take places. Even though there have been several researches documenting the involvement of angiogenesis in tumor growth, invasion and metastasis, but the underlying mechanisms remain unclear because there are several factors have been shown to be involved in this process.

Mast cells are bone-marrow-derived tissue-homing leukocytes which are classified as immune cells of the myeloid lineage. They contain several mediators which are released after being activated. The evidences of intra-tumoral mast cell infiltration and its association with angiogenesis have previously been reported in human and animal neoplasm such as including vascular neoplasms, solid neoplasms and hematological tumors. The role of mast cells associated with tumor pathogenesis remains unclear, therefore further investigation is required.

Sphingosine kinase (SPHK1) is a major substance which is able to generate S1P by phosphorylating of sphingosine. Significantly higher SPHKS1 mRNA levels have been demonstrated in many types of human tumors including lung, colon, breast, ovary, stomach, kidney, uterus, small intestine when compared with the level in normal human tissue

Moreover, a remarkable correlation between SPHKS1 expression and histopathological staging was also noted in cancer patients suffering from colon, gastric and astrocytomas, indicating that SPHKS1 is associated with cancer progression in cancer patients. Moreover, the study by Watson et al. (2010) showed that the significantly positive correlation between the expression of SPHKS1 as well as S1PR-1 with the poor prognosis in patients with breast cancer.

The study focusing on SPHKS1, S1PR-1, mast cells and angiogenesis in benign and malignant type of canine mammary tumor has not been previously documented. It was interesting to focus on evaluating the involvement of these parameters

2. Objectives

2.1. To study the involvement of SPHKS1 expression, S1PR-1 expression, mast cells, and microvessel in canine mammary tumor.

2.2. To explore the correlation among variables including SPHKS1 expression, S1PR-1 expression, mast cell density and microvessel density and also among SPHKS1 expression, S1PR-1 expression to clinical data in canine mammary gland tumor.

3. Methodology

3.1. Specimen collection

Specimens were collected from 60 female dogs, aged between 5-15 years, which were clinically diagnosed of mammary tumor. The clinical data was collected from the hospital records including age, breed, tumor recurrence, metastasis, and survival for two years after surgical removal of mammary gland tumor.

3.2. Histopathologic evaluation

All specimens were processed for histopathological evaluation. The paraffin-embedded tissues stained with Hematoxylin and eosin (H&E) were used for tumor classification into simple adenoma and simple carcinoma, tubular type. Normal control tissue were collected from ten fresh carcasses without macroscopic mammary lesion.

3.3. Mast cell identification

Mast cells were stained by Toluidine blue working solution which was the mixture between 1% toluidine blue solution and 1% sodium chloride prepared according to standard protocol preparation.

3.4. Microvessel identification

Microvessels were identified by immunohistochemistry as previously described (54). Tissues were immunostained with Monoclonal mouse anti-human platelet endothelial cell adhesion molecule (PECAM).

3.5. Identification of sphingosine kinase 1 expression and sphingosine-1 phosphate receptor 1 expression

To examine the expression of S1PR-1, staining procedures were done according to previously described by Akiyama et al., (2008) (1). Tissue were stained with mouse anti-S1P1 receptor antibody and the process was followed by the previous protocol.

To examine the expression of SPHKS1, staining procedures were done according to previously described by Li, et al (2012). Tissue were stained with rabbit anti- SPHK-1 antibody and the process was followed by the previous protocol.

3.6. Digital image capture and Immunohistochemical analysis

All tissue specimens were evaluated under light microscope and Image analysis was performed by using Image Analysis program (NIS-Elements Microscope Imaging Software). Microvessel density, mast cell density and histologically scoring used to evaluate level SPHK-1 or S1P1 expression was performed according to previous study (37). The level SPHK-1 or S1P1 expression was scored into 0-3 score according to staining intensity in cytoplasm of tumor cells. The level SPHK-1 or S1P1 expression was scored into 0-4 score according to the total percentage of immunopositive tumor cells in tumor tissue. For evaluating the correlation between SPHK-1 and S1PR-1 expression, the number of immunopositive cells was assessed by dividing the number of positive cells with the total cell count and multiplying this number by 100. This variables were used for evaluate the correlation level of SPHK-1 and S1PR-1 expression

3.7. Statistical analysis

All statistical analysis was performed using SPSS statistical version 21.0 software. Statistical significance was determined when calculated p value of $\leq$ 0.05. Fisher's exact tests and Spearman's correlation coefficients was used for evaluate relationship between SPHK-1 and S1PR-1 expression with clinical data. To compare mast cell density, microvessel density, SPHK-1 and S1PR-1 positive cells, with two to three intensity score, between simple

adenoma and simple carcinoma or tubular carcinoma, data were calculated and student's t-test was used for data analysis regarding with statistical significance when P values of $\leq$ 0.05. Correlation among these parameters was calculated using Spearman's correlation coefficients.

4 Results

The result showed significant upregulation of SPHK1 expression in cytoplasm of neoplastic cells, increased MCD and MVD density in simple carcinoma, tubular type as compared to tissues of normal mammary tissue and simple adenoma ($p < 0.05$). Positive correlation between SPHK1 expression and MVD, between MCD and MVD and also between SPHK1 expression and metastasis evidence was documented in simple carcinoma, where there were no other correlation of other variables in normal control, simple adenoma and simple carcinoma.

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I would to thank the Thailand Research Funds, the Commissioner of Higher Education Office and Faculty of Veterinary Science, Mahidol University, Nakorn-Prathom for supporting the funds for this research. Special thanks also given to Center of Veterinary Diagnosis, Faculty of Veterinary Science, Mahidol University for providing the instruments and laboratory spaces for carrying this project

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Chapter 1: Background and Rationale

Mammary gland tumor in female dogs is regarded as the second most commonly diagnosed cancer and a major course of cancer-related death. The tumors are commonly found in female dogs aged between 6-7 years old. All breeds can be affected but Miniature poodle, Afghan Hound, Chihuahua, English setter are commonly affected. Most cases, suffering from malignant tumors, are diagnosed at advanced stages with the poor prognosis, resulting from tumor growth and extensive invasion into surrounding tissue, and metastasis of cancer cells to common metastatic sites including lymph nodes, lung, brain, and liver. Angiogenesis is thus an essential process that promotes tumor growth and hematogenous metastatic dissemination, resulting in the poor prognosis of cancer animals. This process has been demonstrated that it is influenced by various factors, for examples, the angiogenic factors, secreted by tumor cells and tumor-infiltrating immune cells, such as VEGF, fibroblast growth factors, and various cytokines. Recent studies *in vivo, in vitro* and human cancers reveal the roles of mast cells, sphingosine-1 phosphate, sphingosine kinase and sphingosine receptor in promoting tumor progression, growth, tumor cell survival and metastasis. These factors exert their roles in human tumors and found to be significantly correlated with clinical outcomes, for examples, prognosis, survival rate, and tumor progression, in patients suffering from malignant tumors. However, no research has been previously reported in veterinary medicine for the roles of these factors associated with clinicopathological parameters. Therefore, I am interested to assess the association among sphingosine kinase-1, sphingosine-1 receptor, mast cell density, microvessel density with histopathological features and clinical parameters in dogs being diagnosed with canine mammary gland tumors. This study might provide the better understanding of pathogenic mechanisms of animal tumors which leads to the advanced research. The approach also enable the identification of biomarkers and signaling protein that might serve as new targets for therapeutic intervention, and also might be designed to improve treatment of canine mammary gland tumor in order to reduce tumor growth, invasion and metastasis and improve prognosis. The results might be useful and applicable to the research of human cancer.

Chapter 2: Literature review

Mammary gland tumors in female dogs have been regarded as the second most commonly diagnosed tumor in which they are documented for being a major cause of cancer-related death whereas they are infrequent in male dogs (6, 60, 61). The common ages of dogs suffering from this tumor range between 6-7 years old while common affected breeds include Miniature poodle, Afghan Hound, Chihuahua, English setter etc. This canine spontaneous tumor shares some similar characteristics with human cancers which the incidence in bitches was found to be three times higher than (7). Various risk factors, including obesity at a young age, hormonal influences, high intake of red meat or home-made diet, influence the prognosis and incidence of this tumor (50, 60). These neoplasms can be classified into benign and malignant neoplasms of which the histopathological features and prognosis are different. For classification of benign and malignant tumors, histological evaluation of Hematoxylin and eosin-stained sections, with regard to tumor types, nuclear and cellular pleomorphism, mitotic index, necrotic area, regional lymph node metastasis, neoplastic invasion into peritumoral tissue and lymphatic vessels, are required for identification. (Goldschmidt, L 2011). Moreover, the tumors have also been classified into different types based on histologic features, in accordance with World Health Organization criteria (1999).The classifications based on histologic features can be categorized into malignant tumor, benign tumor, unclassified tumor, hyperplasia and dysplasia. The malignant tumor of mammary gland includes noninfiltrating carcinoma, complex carcinoma simple carcinoma, special type of carcinoma, sarcoma, carcinosarcoma, carcinoma or sarcoma in benign tumor whereas adenoma, fibroadenoma, benign mixed tumor and duct papilloma. For the more recent classification proposed by Goldschmidet et al. (2010), the benign neoplasms are classified into simple adenoma, intraductal papillary adenoma, ductal adenoma, fibroadenoma, myoepithelioma, complex adenoma, benign mixed tumor while malignant epithelial neoplasms are divided into carcinoma in situ, simple carcinoma, micropapillary invasive carcinoma, solid carcinoma, comedocarcinoma, anaplastic carcinoma, mixed type carcinoma, complex type carcinoma, carcinoma and malignant myoepithelioma,

Malignant tumors are fast growing, highly invasive into surrounding tissue, high recurrence after surgical removal and have highly metastatic potential of neoplastic cells to distant

organs such as lungs regional lymph nodes, kidneys, bone, adrenal glands, brain, liver, pleura and lungs (29, 32). Conventional therapies for mammary gland tumors in veterinary medicine include either the surgical removal, or surgery in combination with chemotherapy (33).

Angiogenesis or neovascularization is a complex process from which the formation of new blood vessels from the preexisting vasculatures take place. There are essential steps in neovascularization including recruitment of endothelial cells from pre-existing blood vessels, proliferation and migration of endothelial cells, maturation and differentiation of capillary sprout (23, 59). This process exerts roles not only in physiological conditions but also in pathological conditions. For examples, physiological angiogenesis is involved in tissue growth, tissue repair, embryonic development, wound healing and collateral formation for improved organ perfusion while pathological angiogenesis is associated with several disorders such as neurodegeneration, hypertension, respiratory distress, and cancer (12).

Angiogenesis has been extensively documented as being fundamental for tumor progression in the form of tumor growth, invasion and hematogenous dissemination of cancer to distant organs (4, 12). As a result of increased oxygen and nutrient supply as well as catabolite removal, this process results in promoting tumor growth. Even though there have been several researches documenting the involvement of angiogenesis in tumor growth, invasion and metastasis, but the underlying mechanisms remain unclear because there are several factors have been shown to be involved in this process. For example, several angiogenic factors, which are produced either from tumor infiltrating leukocytes or from neoplastic cells, serve as powerful mediators promoting intratumorally vascular formation. (12, 13). These activators, for example, vascular endothelial growth factors (VEGF), basic fibroblast growth factor (bFGF), angiogenin, placental growth factor (PGF), interleukin-8 (IL-8), hepatocyte growth factor (HGF), platelet-derived endothelial growth factor (PDEGF), granulocyte colony-stimulating factor (GCSF), tumor necrosis factor (TNF) (46). Among them, VEGF family is a major mediators documented for its involvement in mediating angiogenesis because the expression of VEGF family and their receptors were found in human cancerous tissue (12, 13, 14, 57). Among the VEGF family, VEGF-A, VEGF-B, VEGF-C and VEGF-E, are powerful mediators for promoting new blood vessel proliferation, whereas VEGF-C and VEGF-D exert the major roles in mediating in lymphatic vessel

formation from pre-existing lymphatic vessels called lymphangiogenesis (45). More interestingly, the correlation between the prognosis of patients suffering from cancers and angiogenesis, as evidenced by the number of microvessels per square millimeters or microvessel density, has been previously proposed in several types of cancer such as human breast cancer, lung cancer, prostatic cancer and testicular germ cell tumors (15, 20, 47, 68).

Mast cells (MCs) are bone-marrow-derived tissue-homing leukocytes which are classified as immune cells of the myeloid lineage and distribute throughout several tissue including dermis, submucosa and mucosa of gastrointestinal tract, conjunctiva, respiratory tract, genitourinary tract, choroid plexus of brain in which the close proximity to epithelium, fibroblast, nerves, blood vessel and lymphatic vessels present. They are round-shaped; mononuclear cells contain numerous 0.3 to 0.8 micrometer cytoplasmic metachromatic-granules in which there are large spectrums of bioactive mediators stored. These mediators are released after being activated of mast cell following which a variety of biological action subsequently take place (3, 31). The mast cell-derived mediators can be classified into three types including preformed mediators in secretory granules, newly synthesized lipid mediators and cytokines, chemokines as follows (19,20).

1. Preformed mediators: heparin, histamine, tryptase and other protease
2. Lipid mediators: platelet activating factor, prostaglandin D2, leukotriene
3. Cytokines and Chemokines: TNFα, TGFβ, IL-3, IL-5, IL-16

These mediators are released after being activated by different mechanism through which either Immunoglobulin E (IgE)-dependent or non-IgE dependent pathway mediate. For IgE-dependent activation, it is mediated through the cross-linking between IgE and specific antigen to high-affinity IgE receptor FcϵRI on mast cell surface by which the de novo mediator synthesis and degranulation subsequently cells take place (29). For non-IgE dependent pathway, the activation takes place as a result of several mediators, such as cytokines, complement, adenosine, Toll-like neuropeptide etc., by which the activation will be either positive or negative depending on bindings of ligands to expressed receptor on the mast cell surface (30, 32)

Apart from the involvement in several diseases such as asthma, autoimmune disease, infectious disease, heart disease etc., documentation of mast cell involvement in cancer pathogenesis has also been previously reported. The accumulation of mast cells in closed proximity to lymphatic and blood vessels within tumor parenchyma and and peripheral parenchyma has been observed in animal models and human tumor and the accumulation is found to be associated with prognosis in a various types of tumors (21, 112). The evidences of intratumoral mast cell infiltration and its association with angiogenesis have previously been reported in human and animal neoplasm including vascular neoplasms, solid neoplasms and hematological tumors. For example, reports in human neoplasm include plasmacytoma (41), squamous cell carcinoma (8) and basal cell carcinoma (2) and in animal neoplasm include reported in canine melanomas (41) and canine transmissible venereal tumor (42) and canine mammary gland tumor (69). The mechanisms of mast cell infiltration are relevant to several chemotactic factors synthesized and secreted by tumor cells which exert the action on receptors expressed by mast cells. Focusing on canine mammary tumor, there was the significantly positive correlation between mast cell count and micro-vessel density, indicating the association between mast cells and angiogenesis in canine mammary adenocarcinoma (69).

Phospholipids are regarded as a class of lipid whose structures are composed of two hydrophobic fatty acid and a hydrophilic head containing a phosphate group. Phosphoplipids contain two kinds of sphingolipids and glycerophospholipids. Sphingosine-1-phosphate (S1P), a potent blood-borne lipid mediator, exerts a wide range of functions including cellular proliferation, cellular survival, cell migration, invasion, differentiation, and also plays pivotal roles in control of vascular integrity and angiogenesis (18, 39, 48, 53, 55). Intracellular signals transduced by S1P are mediated through the activation of S1P receptor subtypes, including S1P1, S1P2, S1P3, S1P4, and S1P5, which are found to be expressed in different tissue (5, 30). The roles of S1P and the receptors, associated with the promoting tumor growth, angiogenesis, metastasis and facilitating tumor cell survival have previously been documented (52, 62). The effects of S1P mediated through S1P1 play a role in mediating angiogenesis which consists of a process including endothelial cell migration, proliferation, survival and morphogenesis into capillary-like structure (35; 58). Studies, showing that S1P signaling system, are associated with tumor neovascularization in animal models. Monoclonal anti-S1P antibody, administered repeatedly into

tumor models, in which animals were injected with MDA MB-231 and MDA MB-468 breast carcinoma cells and SKOV3 ovarian cancer cells, effectively inhibit tumor growth (66).

Sphingosine kinase (SPHK1), classified into SPHKS1 and SPHKS2, is a major substance which is able to generate S1P by phosphorylating of sphingosine (64). A study documented that SPHKS1 plays a role in the production of S1P and this action subsequently results in the increased level of S1P in tumor and in blood circulation (43). In vitro study has shown that SPHKS1-expressing tumor cells could release S1P, and subsequently result in endothelial cell migration, and organization into tubular structures. (16). Moreover, a study in murine model of breast cancer, SPHKS1 was found to be able to enhance both formation of blood vessels and lymphatic vessels. (43). Given the increasing evidence which link between the essential roles of SPHKS1associated with cancer pathogenesis, SPHKs are able to exert the function as an oncogenic enzyme capable of exerting anti-apoptosis, proliferation, transformation and tumor cells survival (28, 34). Significantly higher SPHKS1mRNA levels have been demonstrated in many types of human tumors including lung, colon, breast, ovary, stomach, kidney, uterus, small intestine when compared with the level in normal human tissue (16, 26). Previous studies have reported the correlation between the SPHKS1 expression and clinical parameters, such as staging of tumors, survival and tumor progression, in human cancer. The mRNA expression of SPHK-1, in cancer patients suffering from grade IV glioblastoma multiforme and oestrogen-s positive breast cancer, was significantly correlated with poor survival in these patients (56, 65). Moreover, a remarkable correlation between SPHKS1 expression and histopathological staging was also noted in cancer patients suffering from colon, gastric and astrocytomas, indicating that SPHKS1 is associated with cancer progression in cancer patients (27; 36, 37). Further evidence supports that SPHKS1 might possibly be used as a prognostic marker because high SPHKS1expression was significantly correlated with shorter overall survival times of gastric and astrocytomas cancer patients (36, 37). Moreover, the study by Watson et al. (2010) showed that the significantly positive correlation between the expression of SPHKS1 as well as S1PR-1 with the poor prognosis in patients with breast cancer (67).

In vitro and In vivo studies have shown SPHKS might exert the action through FceRI-mediated mast cell degranulation and functional responses (51). Following the degranulation,

mast cell mediators are secreted and induce physiological and pathological response, especially neovascular formation induced by the actions of mast cell angiogenic factors. To our knowledge, there have been no previously studies focusing on SPHKS1, S1PR-1, mast cells and neovascularization in term of comparison and correlation evaluation.

Chapter 3: Objectives

The study focusing on SPHKS1, S1PR-1, mast cells and angiogenesis in benign and malignant type of canine mammary tumor has not been previously documented. It is interesting to focus on evaluating the involvement of these parameters, therefore the objectives of this study are as follows.

1. To study the involvement of SPHKS1, S1PR-1 expression, mast cells and angiogenesis in canine mammary tumor which was classified into a benign type and a malignant type representing as simple adenoma and simple carcinoma, tubular type respectively..

2. To study the correlation among the variables including between SPHKS1 and S1PR-1 expression to clinical data, between SPHKS1 and S1PR-1 expression to mast cell density and microvessel density in each group of simple carcinoma and simple adenoma.

Chapter 4: Research methodology and Results

Methodology

Specimen collection

Specimens of mammary gland tumor and clinical data were collected from 50 female dogs, aged between 5-15 years, which were clinically diagnosed of mammary tumor by veterinarians at Prasu-Arthorn Veterinary hospital, Faculty of Veterinary Science, Mahidol University, Salaya, Nakorn-Prathom, Thailand. All cases were treated with mastectomy and the tissues were submitted to Veterinary Diagnostic Pathology Unite, Veterinary Diagnostic Center, Mahidol University. Moreover, the clinical data was also collected from the hospital records in which age, breed, tumor recurrence, metastasis, and survival was recorded for two years after surgical removal of mammary gland tumor.

Histopathologic evaluation

All specimens were processed for histopathological evaluation. The tissue fixation was performed using 10% buffered formalin and tissues were embedded in paraffin by using Leica automatic tissue processor. The paraffin-embedded specimens were then sectioned for 5 μm thickness on glass slides, deparafifinized in xylene, and dehydrated in graded alcohols. Hematoxylin and eosin (H&E) and Toluidine blue staining were applied to all tissue sections in order to evaluate tumor classfication and mast cell identification. The proposed criteria for histological classification by Goldschmidet et al. (2010) was used to classify the tumor into simple adenoma and simple carcinoma, tubular type for subsequent analyasis.

Mast cell identification

Immunohistochemical stain and toluidine blue stain, were used for mast cell identification. The Toluidine blue stain was performed according to standard protocol. Working solution was made from 1% toluidine blue solution and 1% sodium chloride solution as follows

1% toluidine blue solution

- Toluidine blue power 1 g.
- 70 % alcohol 100.0 ml.

1% sodium chloride solution with fresh preparation

- •Sodium chloride power 0.5 g.
- •Distilled water 50 ml.

Working solution

- •1% toluidine blue 5.0 ml.
- •1% sodium chloride 45.0 ml.

5 μm tissue sections were deparafinized, cleared in xylene and was then rehydrated to descending grades of alcohol. Sections were kept in distilled water for at least five minutes and were then stained with working solution for 1-2 minutes. Dehydration in graded alcohol and mounting were then subsequently done.

Immunohistochemical evaluation

Immunohistochemical identification of microvessels, phingosine-1 phosphate receptor 1, spingosine kinase in paraffin-embedded specimens were carried out in 5-μm thick sections on poly-L-lysine coated glass slide. The paraffin sections were deparaffinized in xylene, graded in different alcohol concentration, phosphate buffered saline (PBS) washed, immunohistochemical stained and were counterstained with Harris's haematoxylin.

Microvessel identification

Microvessels were identified as previously described (54). To block endogenous peroxidase, the specimens were incubated in 0.3% H_2O_2 in absolute methanol for 30 minutes and then treated with 0.4% pepsin in 0.01 mHCl for 30 minutes at 37^{o} C. Monoclonal mouse anti-human platelet endothelial cell adhesion molecule (PECAM) was used as primary antibody with a dilution of 1:20 in Tris-HCl buffer containing 0.5% bovine serum albumin and 0.015 M sodium azide and the specimens were incubated overnight at 4^{o} C and followed by incubating specimens in biotinylated secondary antibody, using labeled streptavidin biotin diluted in PBS for 30 minutes. After PBS washing, the sections were incubated in streptavidin conjugated to horseradish peroxidase in Tris-HCl buffer containing sodium azide 0.015% for 30 minutes. To demonstrate the immunolabeling,

chromogen, using 3, 3 -diaminobenzidine tetrahydrochloride, was applied on the specimens and then counterstained with hematoxylin.

Immunohistochemistry for sphingosine kinase 1 and sphingosine-1 phosphate receptor 1 expression. To examine the expression of S1PR-1, staining procedures were done according to previously described by Akiyama et al., (2008) (1). For antigen retrieval, specimens were submerged in boiling sodium citrate buffer (pH 6), followed by overnight incubating with mouse anti-S1P1 receptor antibody (1:25) at 4°C and incubating specimens in specific secondary antibody was performed. The chomogen, diaminobenzidine tetrahydrochloride solution with H_2O_2, was applied to demonstrate immunolabeling.

For immunostaining of SPHKS1, the procedure was performed as previously documenting by Li, et al (2012). For antigenic retrieval, the specimens were submerged in EDTA antigenic retrieval buffer and put in microwave. To quench endogenous peroxidase activity, treating specimens with 3% hydrogen peroxide in methanol, was followed by incubation with 1% bovine serum albumin to block nonspecific binding. Sections were incubated with rabbit anti- SPHK-1 overnight at 4°C and negative control using normal goat serum. After washing, specimens were treated with biotinylated anti-rabbit secondary antibody (Zymed), followed by streptavidin-horseradish peroxidase complex added and immersed in 3, 3-diaminobenzidine tetrahydrochloride.

Digital image capture and Immunohistochemical analysis

All tissue specimens were evaluated under light microscope and Image analysis was performed by using Image Analysis program (NIS-Elements Microscope Imaging Software) in order to measure area per millimeter squares.

To assess the mast cell density (MCD), the number of toluidine blue-stained (metachromatic) mast cells was performed at 400 x magnification. Selective counting area from at least ten representatively microscopic fields, where there were greatest number of positive cells, was calculated into “the number of mast cells per square millimeter” This parameter represent the mast cell density.

To assess the microvessel density (MVD), which represents intra-tumoral angiogenesis or neovascularization, the evaluation was based on the previous study of angiogenesis in canine

mammary gland tumor (54). At 400x magnification field, at least ten fields per tumor specimens were selected, by which the area of connective tissue stroma was evaluated by Image analysis program. The number of vessels per square millimeters were calculated.

To assess the level of SPHK-1 and S1PR-1 expression, at least ten fields per tumor specimen, at 400x magnification, were selected. Scoring method was based on the previous study of SPHK-1 and S1PR-1 expression in human cancer (37). For each specimen, the level of expression was evaluated by histologically scoring. The level SPHK-1 or S1P1 expression was scored into 0-4 score including 0 score: no positive tumor cells, 1 score: less than 10% positive tumor cells, 2 score: 10-35% positive tumor cells, 3 score: 35-70% positive tumor cells, 4 score; more than 70% positive tumor cells. To determine staining intensity, the criteria includes 0 = no staining, 1 = weak staining (light yellow), 2 = moderate staining (yellow brown), and 3 = strong staining (brown). Staining index was calculated as the result of intensity score and proportion of positive tumor cells. The index of more than six was used to defined tumor with high SPHK-1 and S1PR-1 expression while low tumor with low expression was defined as the score less than four.

To evaluate the correlation level of SPHK-1 and S1PR-1 expression, the number of immunopositive cells were examined for each marker and was calculated by dividing the number of positive cells with the total cell count and multiplying this number by 100.

Statistical analysis

All statistical analysis was performed using SPSS statistical version 21.0 software. Statistical significance was determined when calculated p value of $\leq$ 0.05. To evaluate the relationship between expression of SPHK-1 and S1PR-1 expression with clinical characteristics, the analysis was performed by Fisher's exact tests and Spearman's correlation coefficients. To compare mast cell density, microvessel density, SPHK-1 and S1PR-1 positive cells between control group, simple adenoma and simple carcinoma, data was calculated as mean$\pm$standard deviation (SD) and student's t-test was used for data analysis regarding with statistical significance when P values of $\leq$ 0.05. Correlation among these parameters was calculated using Spearman's correlation coefficients.

Results

Histopathologic and Immunohistochemical evaluation

The mammary mass specimens were collected from 50 female dogs, aged between 3-14 years old, the mean age was 7± 0.3 years. Weight was between 7 to 40 kilograms (kg) and the mean age was 15± 1.2 kg. There were several breeds affected with mammary tumor including fourteen Thai dogs, eight Mixed breed dogs, six Poodle breed dogs, five Boxer breed dogs, nine Bangkaew breed dogs, five shih tzu breed dogs, three Golden retriever breed dogs. In this study, Thai breed dogs were found to be the most affected breeds suffering from mammary gland tumor. All dogs were treated with mastectomy and specimens including mammary tumor specimens as well as control specimens collected from necropsy were submitted for histological evaluation. Histological evaluation revealed twenty dogs with simple adenoma, thirty dogs with simple carcinoma, tubular types and ten dogs with normal mammary tissue serving as control groups.

Clinical data from hospital records including metastasis, two years survival after mastectomy, tumor recurrence were recorded. For metastasis data, nine from thirty dogs (30%) with simple carcinoma have tumor metastasis whereas no records showing metastasis in simple adenoma (0%) and control groups (0%). For survival after the treatment, five dogs with simple carcinoma (16.7%) died after six months to fifteen months whereas no dogs with simple adenoma died after mastectomy. For tumor recurrence, four dogs with simple carcinoma (13.3%) have recurrent mammary mass twelve to sixty weeks after surgery.

In this study, mammary mass classification was based on histological evaluation. The benign and malignant mammary tumor were classified according to the criteria based on histological findings which include nuclear and cellular pleomorphism, mitotic index, invasiveness of neoplastic cells into lymphatic and blood. All specimens were histologically divided into twenty samples of simple adenoma, thirty samples of simple carcinoma, and ten samples of normal control collected from necropsy. The simple adenoma and simple carcinoma, tubular types represent a benign tumor and a malignant tumor respectively.

Simple adenoma was a well demarcated mass in which there are cuboidal to columnar tumor cells characterized by cells with central round to oval nuclei with small central nucleolus finely stippled nuclear chromatin, and moderate amount of eosinophilic cytoplasm. The arrangement of tumor cells was present as a single layer which forms tubular structures within scant to moderate degree of fibrovascular stroma. The tumor cells exhibit minimal degree of anisocytosis, anisokaryosis and mitotic figures. The histologic findings are shown in Figure 1A

Simple carcinoma, with tubular carcinoma subtype was histologically characterized as a malignant neoplasm with variable neoplastic cell morphology. Neoplastic cells variably showing normochromic, hypochromic or hyperchromic nuclei with either single, large nucleoli or multiple, small nucleoli and eosinophilic cytoplasm were seen. The neoplastic cells were arranged in a tubular fashion lined with one to two layers of neoplastic cells while intertubular stroma consists of blood vessels and fibroblast. High mitotic figures and anisokaryosis are also noted. Microscopic findings are shown in Figure 1B

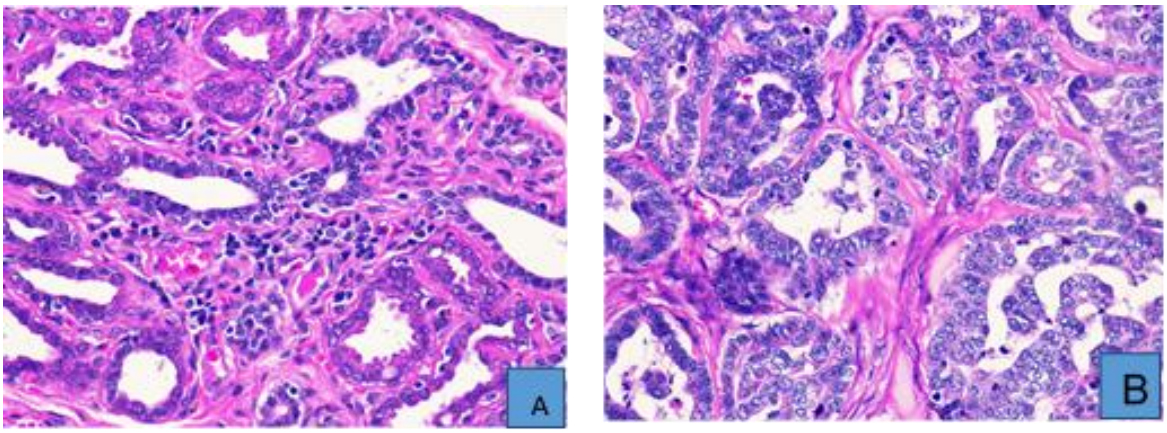


Figure 1: Histological classification of canine mammary tumor showing a benign type and a malignant type as follows: (A) Simple adenoma (B) Carcinoma, tubular type. Tissue stained with Hematoxylin & Eosin

For mast cell density evaluation, Toluidine blue stain, an acidophilic metachromatic dye. All toluidine blue-positive mast cells appear as round to oval-shaped cells with red-purple granules in cytoplasm (Figure 2) with regard to metachromatic stain while the background was present as orthochromatic staining with blue-violet colored stain (Figure 2). Mast cell infiltration were observed within the intratumorally connective tissue stroma as well as on the periphery of tumor tissue. The higher number was present in tubular carcinoma (Figure 2A) as compared with simple adenoma (Figure 2B). Statistical analysis revealed the average number of mast cells per mm^2, which represents as MCD, were 6.3±2.3, 10.2±3.6, and 43.2±12.1 in the tissue of normal control, simple adenoma and tubular carcinoma respectively. MCD was significantly higher in tubular carcinoma as compared to those in control group and simple adenoma group (Figure 3).

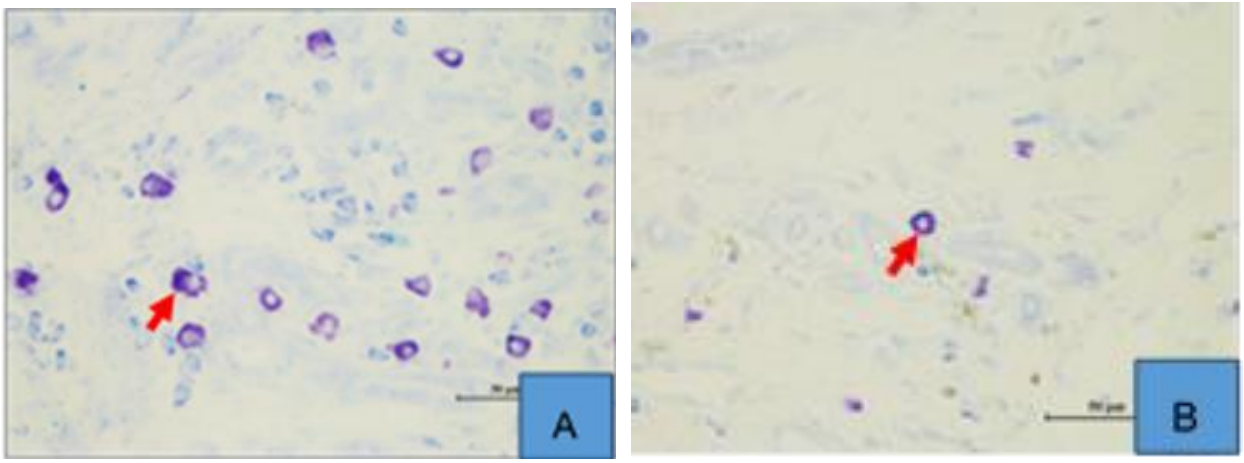


Figure 2: Mast cells (red arrows) in the connective stroma at periphery of tumor tissue (A) simple carcinoma, tubular type and (B), simple adenoma tissue. Tissues stained with Toluidine blue.

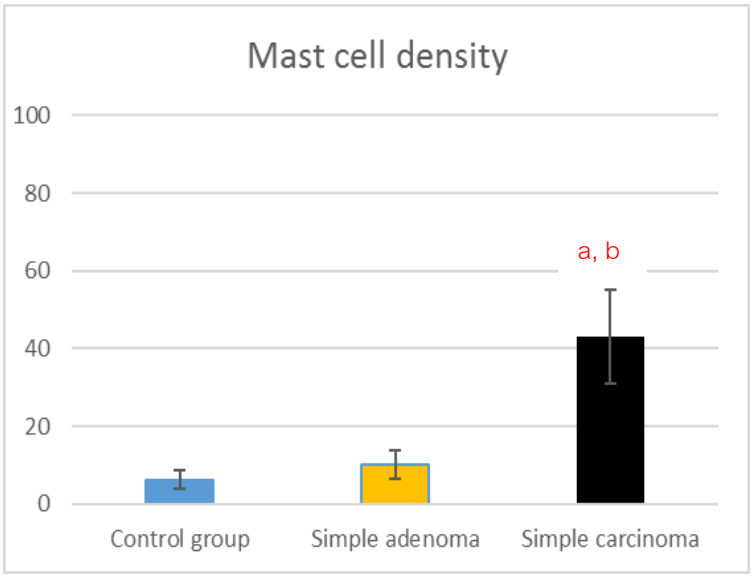


Figure 3: Quantitative analysis of mast cell density in control groups, simple adenoma and simple carcinoma. a and b, defined as statistical significance when compared with the control and simple adenoma respectively.

Using immunohistochemical stain, microvessels were identified due to the criteria showing that every immunolabeling endothelial cells are separated from adjacent microvessels, tumor cells, and other connective tissue elements and were counted as a single microvessel (Figure 4, arrow). Significantly higher number of microvessels in simple carcinoma sections (Figure 4A) as compared with simple adenoma (4B) were documented. The mean MVD$\pm$standard deviation was 20.1$\pm$5.2, 28.4$\pm$8.2 and 56.6$\pm$12.8 per mm^2 in control group, simple adenoma and tubular carcinoma respectively. The significantly higher of MVD was present in simple carcinoma. (Figure 5)

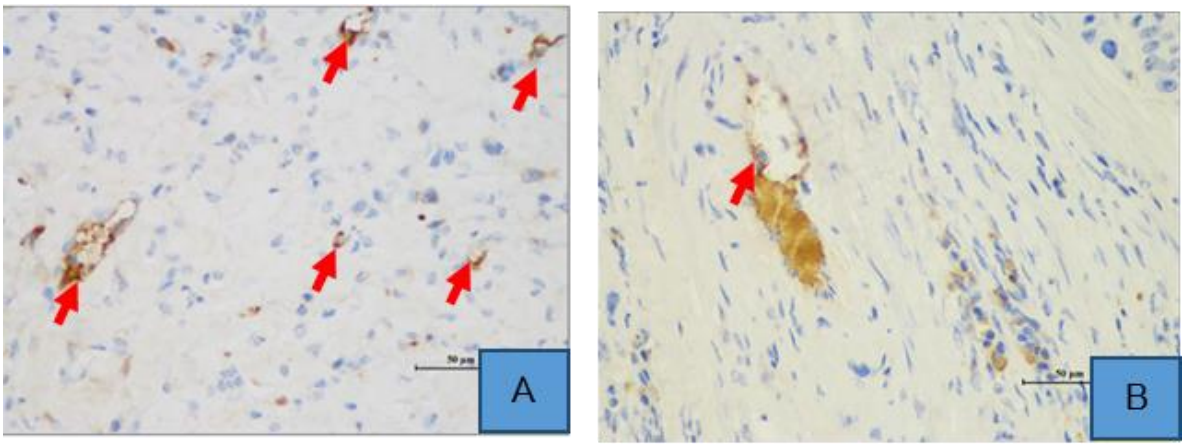


Figure 4: Microvessels (red arrows) on the peripheral area of (A) tubular carcinoma tissue and (B) simple adenoma tissue. Tissue immunohistochemially stained antibody labelled for PECAM.

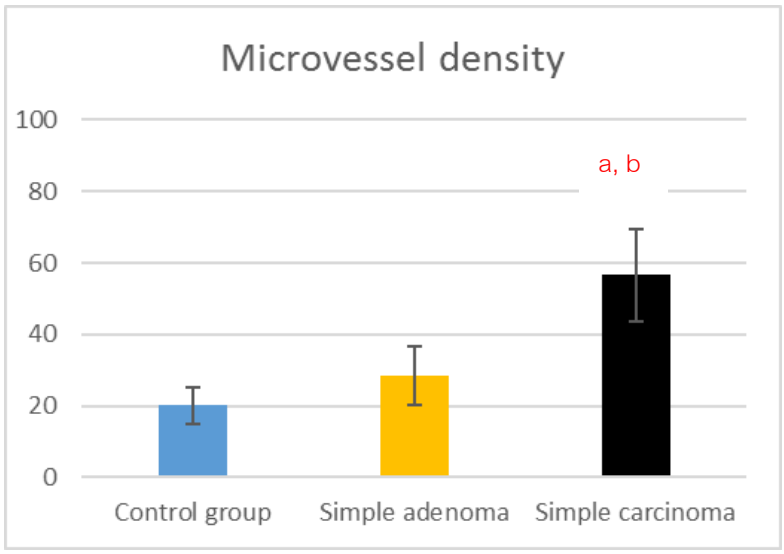


Figure 5: Quantitative analysis of microvessel density in normal mammary tissue, simple adenoma and simple carcinoma. a and b, defined as statistical significance as compared with the control group and simple adenoma respectively.

For SPHK1 and S1PR-1 localization, the staining intensity, scored from zero to three, were used for evaluating level of intracytoplasmic expression as shown in Figure 6, red, blue, black, yellow arrows was pointed for score 0, 1, 2, 3 respectively. The strong upregulation of SPHK1 in simple carcinoma was evidently observed as the higher number of immunopositive cells with score two to three staining intensity whereas no stained or weakly immune-stained cells, with score zero to one, and was observed in control group and simple adenoma group. Staining intensity for the expression of SPHK1 in simple adenoma and simple carcinoma was shown in Figure 6

The immunopositive cells with high SPHK1 and S1PR-1 expression level were defined as the cells with high intensity or with score two to three. Only these positive cells, with score two to three, were counted and presented as the percentage$\pm$ standard deviation of SPHK1 positive cells and S1PR-1 positive cells. The mean percentage of SPHK1 positive cells are 42$\pm$12.2, 16$\pm$5.6 and 10$\pm$ 4.6 in simple carcinoma, simple adenoma and normal control group respectively. Therefore, overexpression of SPHK1 in simple carcinoma was evidently present as compared to those in simple adenoma and normal control.

The mean percentages of S1PR-1 positive cells were 7$\pm$0.6, 5$\pm$0.8 and 4.1$\pm$0.6 in simple carcinoma, simple adenoma and normal control group respectively. The low expression of S1PR-1 in all groups was observed and there were no statistically significant differences among these groups. The analysis of SPHK1 and S1PR-1 expression level in mammary tumor groups and control group was present in Figure 7.

For the analysis of correlation among variables, the SPHK1 and S1PR-1 expression with clinical data and also with MCD and MVD were determined with appropriate statistics in simple adenoma and simple carcinoma group. For the SPHK1, S1PR-1 expression, MCD and MVD in simple carcinoma group, there were positive correlation (r=0.36) between MCD and MVD although not significant (p=0.121). Moreover, positive correlation between SPHK1 expression and MVD was also documented although not significant as well (r=0.29, p=0.124). Otherwise, no significant correlation among variables, including between SPHK1 and S1PR-1 (r=0.08, p=0.472), SPHK1 and MCD (r=0.15, p=0.286), S1PR-1 expression and MCD (r=0.12, p=0.235), and between S1PR-1 expression and MVD (r=0.09, p=0.452). For relationship of SPHK1 with the clinical data including age, breed, tumor recurrence, metastasis, survival, SPHK1 expression was the only one factor which was found to be correlated with metastasis evidences (p=0.04) of dogs with simple carcinoma whereas this correlation was not present in simple adenoma group. There was no significant

correlation between S1PR-1 expressions to clinical parameters because p-value were more than 0.05.

In simple adenoma group, there was no any correlation between all variables. No correlation including between MCD and MVD (r=0.08, p=0.624), SPHK1 expression and MVD (r=0.02, p=0.724), SPHK1 and S1PR-1 (r=0.03, p=0.721), SPHK1 and MCD (r=0.03, p=0.724), S1PR-1 expression and MCD (r=0.04, p=0.662), S1PR-1 expression and MVD (r=0.02, p=0.710). No clinical data was found to be associated with SPHK1 expression and S1PR-1 expression due to the p-value was more than 0.05.

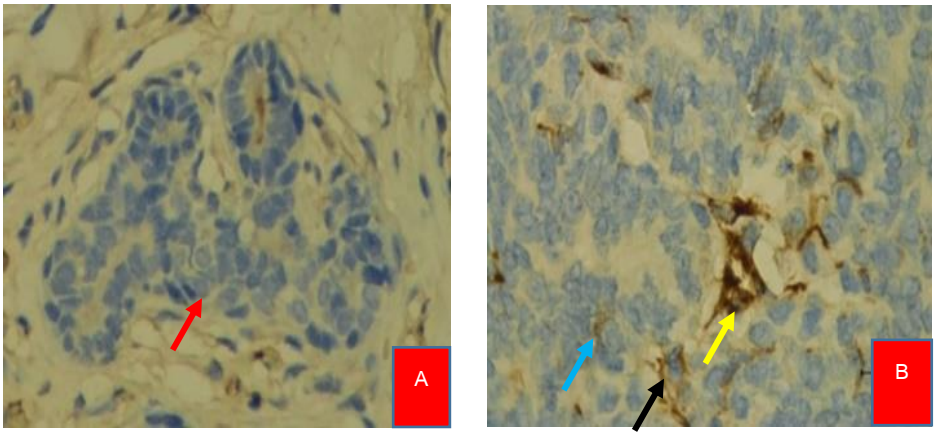
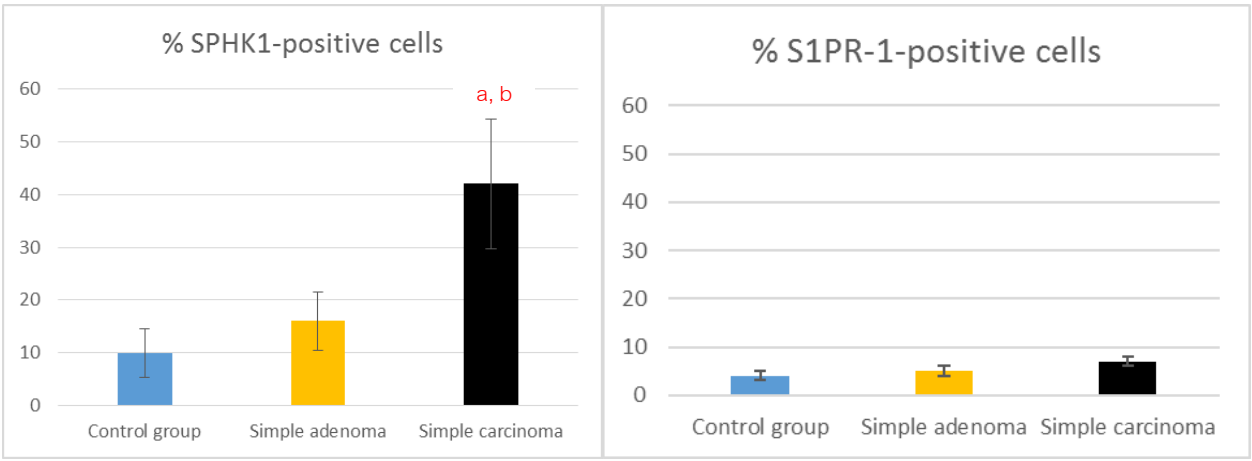


Figure 6: Representative images show the immune-staining intensity level of SPHK-1 in (A) simple adenoma (A) and (B) simple carcinoma, tubular type. Staining intensity was scored and presented as 0 = no staining (red arrow), 1 =weak staining, light yellow (blue arrow), 2 = moderate staining, yellow brown (black arrow), and 3 = strong staining (yellow arrow).



A B

Figure 7 Percentage of positively stained for SPHK1 expression (A) and percentage of positively stained cells for S1PR-1 expression (B). a and b, defined as statistical significance as compared with the control and simple adenoma respectively.

Chapter 5: Discussion

Mammary neoplasms are the most common neoplasm in female dogs however the pathogenic mechanism remains unclear and is still required further investigation. The classification systems based on histologic evaluation were proposed in 1974 and 1999 while the classification used in this study was based on histologic classification in 2010 by Goldschmidt et. al. (70). Mammary gland tumor is categorized into benign neoplasms, malignant epithelial neoplasm, malignant epithelial neoplasm with special types, malignant mesenchymal neoplasms, carcinosarcoma, hyperplasia and dysplasia, neoplasms of nipples and hyperplasia/ dysplasia of the nipple. At the initial process, several tumor subtypes with clinical data were selected for the experiment, finally simple adenoma and simple carcinoma, tubular types were collected because there was sufficient tissue availability as well as the tissue sections suitable for analysis, in which the tissue contains intra & peripheral stromal area valuably used for determining microvessel and mast cell density.

In this study, the pathogenesis associated with mammary gland tumor was evaluated by mainly focusing on SPHK1, SIP-R1, mast cells, neovascularization in simple adenoma and simple carcinoma, tubular type. Mast cells are immune cells, containing several mediators, of which have been found to be associated with pathogenesis in various types of human and animal cancer. In this study, Toluidine blue and immunohistochemical stain, as sensitive staining for mast cell identification were used for MCD analysis as previously reported. (71). For microvessel identification, several vascular markers has been used for MVD evaluation including VEGF, CD31, CD34, CD 15 (72). In the present study, anti-PECAM immunolabelling was used for microvessel identification as previously described (54) and it has been regarded as a good staining due to lesser nonspecific staining with clear vessel identification.

Significantly higher MVD in simple carcinoma, tubular type as compared to those in simple adenoma and normal mammary tissues was documented. The result was consistent with previous study showing the increased MVD was associated with malignant tumor (73) The increased number of mast cells within intra-tumoral and peripheral supporting stroma was also present in simple carcinoma and simple adenoma. MCD in simple carcinoma was also

significantly higher than those seen in simple adenoma and normal mammary tissues. The result was consistent with the previous study (69). Woldemeskel and Rajeev (2010) reported the association between mast cell and angiogenesis in canine mammary gland tumor, cutaneous hemangioma and hemangiosarcoma. The association between mast cell count and MVD in adenocarcinoma evidenced by the positive correlation between these two parameters although the correlation was not statistically significant. Likewise, the role of mast cells in association with angiogenesis was also documented in oral squamous cell carcinoma (OSCC). The mean MCD was comparatively higher in oral OSCC as compared to normal oral mucosa and a significant correlation been MCD and MVD was also documented (73). As mentioned before, angiogenesis or neovascularization is an important process by which new capillary blood vessels form from preexisting vessels. This process is involved in both physiological and pathological conditions. For pathological conditions, cancer angiogenesis, particularly in malignant tumor, has been documented for the involvement not only in tumor progression, but also in metastatic spread of neoplastic cells (74). Previous studies reported that the changes associated with tumor microenvironment possibly result from the interaction among neoplastic cells, infiltrating immune cells and extracellular matrix. For example, endothelial cells in fast-growing cancer are found to be more active as a result of being activated by several mediators such as TNFα, IL-8, prostaglandin E1 and E2, and VEGF (75, 76). In this study, an increase in microvascular formation, as evidenced by high MVD in simple carcinoma, is suggested to be an outcome of pro-angiogenic and angiogenic mediators produced by neoplastic cells and infiltrative leukocytes within the connective tissue of the tumor. Mast cell produces several pro-angiogenic and angiogenic mediators such as histamine, heparin, chymase, tryptase, basic fibroblast growth factor (bFGF), VEGF, IL-8 (24). Histamine and tryptase are well known as major mediators of mast cells and both are found to act as activators for blood vessel formation. Histamine mediates neovascular formation though the action on Histamine receptor 1 and 2 (51) while tryptase induces endothelial cell proliferation (51), enhance vascular formation as well as degrading connective tissue matrix by which spaces in tumor parenchyma are created and facilitate neovascular formation (51). In addition to various kinds of pro-angiogenic factors, including VEGF, bFGF, angiogenin, placental growth factors, IL-8, hepatocyte growth factor and TNF, are produced by neoplastic cells and these mediators also exert the roles on blood vessel formation

as well (68). Therefore, it is speculated that the increased neovascularization, evidently present in simple carcinoma, results from the synergistic actions not only from mast cells but also from neoplastic cells. Apart from increased neovascularization, increased infiltration of mast cells was also present in the study. The mast cell recruitment in connective tissue stroma is mediated by chemoattractant factors produced by neoplastic cells and these mediators exert their action on mast cell receptors. Stem cell factor (SCF) is a ligand for CD117 or KIT receptor which is tyrosine kinase receptor expressed by mast cells. SCF is found to be a major mediator by which its activation on KIT pathway exert a role on mast cell maturation, migration and survival (37Fใน drive not paper.) Prostaglandin E2 and histamine actions through EP2 receptor and VEGFs are functional through VEGF receptor-1 (VEGFR-1) and VEGFR-2 (77).

SPHK1 is an enzyme which is involved in the sphingolipid metabolic pathway. This enzyme is responsible for the production of sphingosine-1-phosphate (S1P) through catalyzing the phosphorylation of sphingosine. Two main isoforms of SPHK include SPHK1 and SPHK2 of which these enzymes exert different functions affecting on biological processes. SPHK1 is found to be a key enzyme which functions in promoting cell growth, tumorigenesis and angiogenesis (26, 39). Where SPHK2 plays a role in apoptosis promotion and cell growth interference (26). Therefore, expression of SPHK1 and its receptor was focused on this study in order to evaluate with other parameters associated with angiogenesis. The distribution of these enzymes is found to vary in different tissue. SPHK1 mRNA expression is high in lung, spleen and peripheral blood leukocytes, whereas the highest expression of SPHK2 is documented in the liver, brain and heart (78). Using immunohistochemical stain, the weak to moderate SPHK1 staining was seen in human liver, spleen, intestine and testis. S1PR-1 is a G-protein couple receptor which binds with S1P as a high-affinity ligand. It is present as an abundant transcript in endothelial cells (43, 48) and mediate the function in regulating endothelial cell cytoskeletal structure, migration, capillary-like network formation and vascular maturation. For cancer research by Kluk and colleaques (2015), involvement of S1PR-1 as a functional receptor was documented that the high expression of S1PR-1 in neoplastic cells of classical Hodgkin lymphoma (78).

In this study, immunohistochemical analysis was consistent with previous studies showing the involvement of SPHK1 in breast cancer (43, 67). The significantly higher expression levels of SPHK1 in simple carcinoma was present as compared with normal mammary tissue and simple

adenoma. The negative staining in normal mammary tissue, negative to weak staining in simple adenoma and higher level of positive staining in simple carcinoma was observed. In addition, the relationship with clinical data, MVD and MCD showed the positive correlation of SPHK1 with metastasis and MVD although not statistical significance where there was no correlation with age, survival, and MCD in normal group, simple adenoma and simple carcinoma.

This suggest that overexpression of SPHK1 is a feature in mammary carcinoma and might partly represent as a predictive marker for metastasis of the disease. The result is partly consistent with previous reports showing the involvement of SPHK1 in several malignant phenotypes including glioblastoma multiforme and colon cancer (27, 28, 65). Moreover, the correlation with clinical data was consistent with previous study documenting that lower metastasis was observed in breast cancer patients whose cancer tissues contain low level of SPHK1 expression (43). The possible explanation might be associated with S1P level in cancer patients which is found to be is associated with SPHK1 expression in SPHK1/S1P axis. The breast cancer patients with lymph node metastasis of which poor prognosis take places (79). This possibly results from increased S1P level, by which lead to new blood vessel formation in the tumor. Therefore, SPHK1 is likely to be a predisposing factor which promote cancer metastasis as evidenced by the positive correlation in this study. Moreover, the correlation of SPHK1 and MCD was not statistically significant, therefore MCD might be influenced by chemoattractants, such as SCF, by which mast cells are attracted to infiltrate in tumor stroma. SPHK1 exerts the action on mast cell activation which subsequently leads to mast cell degranulation and tissue response due to mast cell mediators (51).

For the S1PR-1 expression level, negative to very weak expression, as shown by either negative or weakly-stained cytoplasm of neoplastic cells, was observed in normal mammary gland ,simple adenoma and simple carcinoma. In addition, there was no significant correlation between S1PR-1 expression level with MVC, MCD and all clinical data. As previously mention, S1PR-1 is commonly present in endothelial cells, therefore the expression of this receptor is reported in human vascular neoplasm (1). Rodriguez and colleagues (2015) showed the higher expression level of S1PR-1 in hemangiosarcoma cells was present when compared to nonmalignant endothelial cells (80).

Chapter 6: Conclusion

This study indicates an increase in the expression of SPHK1 in neoplastic epithelial cells of canine simple carcinoma, a subtype of malignant mammary tumor and also an increased in mast cell infiltration as well as proliferation of microvessels in connective tissue stroma. Tumor cells expressing SPHK1 was found to be associated with increased MCD and MVD and also with clinical outcome particularly metastatic spread of neoplastic cells. Tumor microenvironment might be under the influences of these factors in association with other factors such as the release of mediators by tumor cells by which leading to poor prognosis outcomes in animal suffering from malignant mammary neoplasm. Therefore, therapeutic intervention focused on these factors which might be used as targets for the management of animals suffering from mammary carcinoma. At present, several drugs focused on inhibition of mast cells and spingosine pathways are in the process used for cancer therapies.

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