A THESIS FOR THE DEGREE OF MASTER

TRPM7 as a potential diagnostic and prognostic marker of canine mammary gland tumors

개 유선 종양의 진단 및 예후 마커로서의 TRPM7

2020년 8월

서울대학교 대학원

수의학과 임상수의학 전공

이 슬 지

TRPM7 as a potential diagnostic and prognostic marker of canine mammary gland tumors

유선 종양의 진단 및 예후 마커로서의 TRPM7

지도교수 김 완 희

이 논문을 수의학 석사 학위논문으로 제출함

2020년 5월

서울대학교 대학원
수의학과 임상수의학 전공
이 슬 지

이슬지의 석사 학위논문을 인준함
2020년 7월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

TRPM7 as a potential diagnostic and prognostic marker of canine mammary gland tumors

Supervised by

Professor Wan Hee Kim

Seulji Lee

Major in Veterinary Clinical Science

Department of Veterinary Medicine

Graduate School of Seoul National University

ABSTRACT

Transient receptor potential melastatin 7 (TRPM7) is a Ca^{2+} and Mg^{2+} permeable cation channel that contains a protein kinase domain. The aim of this study was to determine TRPM7 expression in 57 benign and malignant malignant mammary gland tumor (MGT) tissues of dogs using immunohistochemistry (IHC)

and evaluate its correlation with Ki-67 poliferation index and clinicopathological features. Ki-67 expression (≤15%, >15% of cells) and tumor size (≤3 cm, >3 cm) were assessed. IHC analysis showed that TRPM7 is expressed in the cytosol of cancer epithelial cells. Moreover, TRPM7 was overexpressed in 3/36 (8.34%) benign MGTs and 14/21 (66.67%) malignant MGTs. Furthermore, TRPM7 expression was significantly associated with a higher Ki-67 and larger tumor size in highly malignant tumors. Survival curves analysis indicated that high TRPM7 expression is significantly associated with poor disease-free and overall survival. Our results demonstrate that TRPM7 channels are overexpressed in canine MGTs and TRPM7 overexpression is positively correlated with clinicopathological parameters. In conclusion, high TRPM7 expression can be a valuable diagnostic and prognostic biomarker for canine MGT.

Keywords : Dog, Ion channel, Immunohistochemistry, Mammary gland tumor, Transient receptor potential melastatin 7

Student Number : 2016-21780

CONTENTS

LIST OF ABBREVIATIONS

CMTs	**Canine mammary gland tumors**
DAB	**Diaminobenzidine**
DFS	**Disease-free survival**
IHC	**Immunohistochemistry**
OS	**Overall survival**
TRP	**Transient receptor potential**
TRPM7	**Transient receptor potential melastatin 7**

LIST OF TABLES

LIST OF FIGURES

1. Introduction

Canine mammary gland tumors (CMTs) account for 70% of all tumors in intact female dogs (Withrow et al., 2013; Johnston, 2018), and can be benign or malignant in nature. CMTs are hormone-dependent and might recur after surgical removal or metastasize to other organs, in particular, the lymph node and lungs (Nassiri et al., 2018). The prognostic factors of cancer include histological type, histological malignancy grade, mode of tumor growth, lymph node status, and tumor size (Santos et al., 2013). Deregulation of Ca^{2+} homeostasis has been implicated in mammary gland disease (Lee et al., 2006; VanHouten, 2005; Sergeev, 2005; Lee et al., 2002). Moreover, Ca^{2+} and Mg^{2+} ion channels play an important role in cell proliferation, differentiation, apoptosis, and oncogenesis. (Dhennin-Duthille et al., 2011; Guilbert et al., 2009).

Transient receptor potential (TRP) is a plasma membrane ion channel that regulates the permeability of Ca^{2+} and Mg^{2+} across the plasma membrane of animal cells (Sergeev, 2005). TRP channel activity is important for essential hallmarks of carcinogenesis. Therefore, TRP channels have not only been suggested as clinical markers, but also as promising anticancer targets in recent years (Rodrigues, Sieglitz, & Bernardes, 2016). TRP channels were first discovered in a trp-mutant strain of the fruit fly Drosophila, and are categorized into six subfamilies based on their amino acid sequences: the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin), and TRPP (polycystin) channels (Park et al., 2014). Dhennin-Duthille et al. (Dhennin-Duthille et al., 2011) reported that increased expression of TRP channels is a useful biomarker for the diagnosis, prognosis, and/or

treatment of human breast ductal adenocarcinoma. Accumulating evidence has indicated that increased expression of TRP channels can be used as a biomarker for several human malignancies (Chen et al., 2014; Bödding, 2007; Gkika and Prevarskaya, 2009). TRPC, TRPM, and TRPV expression was shown to be correlated with malignant growth and cancer progression (Ouadid-Ahidouch et al., 2013). Furthermore, an increased expression of transient receptor potential melastatin 7 (TRPM7) channel is correlated with breast cancer progression and metastasis (Middelbeek et al., 2012). Another study showed that siRNA-mediated knockdown of TRPM7 expression in most cell types impairs biological functions, highlighting the importance of TRPM7 expression in these cells (Guilbert et al., 2009). A previous study demonstrated the presence and distribution of TRPM7 channels in the normal mammary gland tissue using RT-PCR, immunohistochemistry and western blot (Lee et al., 2020).

The role of TRPM7 in human breast cancer development is well known (Yee et al., 2014, Lee et al., 2006; VanHouten, 2005; Sergeev, 2005; Lee et al., 2002). However, to our knowledge, no study has yet demonstrated TRPM7 expression in CMTs. Hence, this study was conducted to determine the expression of TRPM7 channel in CMT tissues using immunohistochemical (IHC) staining. The correlation between TRPM7 and proliferative index (Ki-67) and clinicopathological features of dogs was also evaluated. The results of this study provide valuable insights into the use of TRPM7 as a prognostic and diagnostic marker of CMTs.

1. Materials and Methods

1.1. Tissue samples

A total of 57 tissue samples from dogs diagnosed with CMTs at Seoul National University Veterinary Medical Teaching Hospital between 2010 and 2019 were used in this study. Of these, 21 and 36 were from malignant and benign tumors, respectively. Informed consent was obtained from the owners for the collection and use of tissue samples. All experiments were approved by the Institutional Animal Care and Used Committee of Seoul National University (SNU-190314-6). The data used in this study included only those for animals with mammary gland tumors (primary lesion) after being diagnosed with fine needle aspiration or biopsy prior to surgical removal. All CMT tissues were fixed in 10% neutral buffered formalin for 48 h at room temperature and embedded in paraffin blocks. Thereafter, 4-μm sections were cut and slides were stained with hematoxylin-eosin for diagnostic purposes. Each slide was evaluated under a microscope and classified according to the diagnostic criteria recommended by the World Health Organization classification and grading for CMTs (Goldschmidt et al., 2016). Mammary gland carcinoma grades were classified, based on tubule formation, nuclear polymorphism and mitotic count: Grade I (low), Grade II (intermediate) and Grade III (high). Ki-67 proliferative index (≤15%, >15% of the cells) and tumor size (≤3 cm, >3 cm) were also evaluated. In this study, one tumor was selected per animal. Animals with a single mammary gland tumor as well as those with multiple tumors were included in this study. In addition, animals with mammary gland tumors and other malignancies detected by various

screening tests prior to surgical removal or those already undergoing chemotherapy were excluded.

1.2. Immunohistochemistry

The paraffin-embedded tissues were sectioned at 4-μm intervals using a microtome (Leica Microtome HM355S, Plymouth, MN), deparaffinized in xylene twice for 5 min each and rehydrated in graded alcohol (100% twice, 95%, 90%, 80%, and 70% once for 3 min each). Antigen retrieval was carried out using a 2100-retriever pressure cooker (PickCell Laboratories, Amsterdam, Netherlands) in a 10mM citrate acid (pH 6) buffer for 20 min. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 for 30 min. The sections were treated with normal horse serum (S-2012, Vector, CA, USA) for 20 min to block non-specific binding and incubated overnight at 4 ℃ with goat anti-TRPM7 antibody (1:300; ab729, Abcam, Cambridge, USA) and rabbit anti-Ki-67 polyclonal antibody (1:500; PA5-19462, Invitrogen Ltd, Paisley, England). The sections were subsequently incubated with secondary antibodies HRP anti-goat IgG (ImmPACTTM, Vector, CA, USA) and HRP anti-rabbit IgG (ImmPACTTM, Vector, CA, USA) for 1 h. The slides were incubated in 3,3′-diaminobenzidine tetrahydrochloride (ImmPACTTM diaminobenzidine (DAB) peroxidase substrate kit, Vector, CA, USA) for 90 s and the reaction was stopped by immersion in distilled water. Negative control samples were incubated in the absence of the primary antibodies to rule out non-specific binding by the secondary antibodies. The tissue sections were counterstained with

Mayer's hematoxylin, dehydrated in graded alcohol, and cleared in xylene. The slides were washed with PBS between each procedure. Immunostained slides were scanned using an Olympus BX51 microscope (Olympus, Japan) with appropriate light filters (Tucsen, Fuzhou, China).

1.3. Qunatitiation of IHC staining

IHC results were analyzed using Aperio ImageScope version 12.3.0.5056 (Aperio, Vista, CA, USA). IHC slide images were analyzed using the Aperio program. These algorithms used color de-convolution to perform stain separation in order to separate DAB from the hematoxylin counterstain. The algorithm to determine the intensity of cytoplasmic and nuclear staining for each slide was used to calculate the staining intensity and percent of target labeled by digitally analyzing the color intensity. The output staining intensities ranged from 0 (negative) – 3 (strong positive) and were correlated with conventional manual scoring methods (Singh et al., 2014). Aperio Cytoplasm V2 algorithm was used to analyze cytoplasmic positivity of TRPM7 expression, based on TRPM7 in human breast cancer criteria (Putluri et al., 2014). Staining intensities for TRPM7 expression ranged from 0–3 and were correlated with conventional manual scoring methods: 0 (negative), +1 (weak positive), 2+ (moderate positive) and 3+ (strong positive). Scores of 2+ and 3+ represented TRPM7 overexpression and the percentage of cases overexpressing TRPM7 was calculated.

Aperio Nucelar V9 algorithm was used to analyze nuclear positivity of Ki-67. An algorithm for analyzing nuclear immunoreactivity was used to measure the

percentage of immunoreactive cells (Dowsett et al., 2011; Gasperetti et al., 2011).All positively stained cells in the whole cell area were counted and the fraction of positive cells was calculated as the number of positive cells/1000. High Ki-67 index value was defined as ≥15% independent of nuclear staining intensity. A ≥15% cut-off threshold was used in accordance with Kadthur et al. (Kadthur et al., 2011), in which a comprehensive standardized statistical analysis was used to form low and high-risk groups based on appropriate cut-off value to indicate the prognosis of CMTs.

1.4. Follow-up data

Dogs with CMTs were followed up for at least for 1 years. All the dogs were followed up post-surgically via telephonic conversation with the clients and referral hospital (minimum 23 days, maximum 3206 days). Disease-free survival (DFS) was defined as the interval (months) from primary surgical treatment to the date of detection of the first local recurrence or development of distant metastases. Overall survival (OS) was calculated from the date of primary surgical treatment to time of death from the cancer. Assessment of metastasis and recurrence of tumors were carried out by physical examination, fine needle aspiration, and thoracic radiograph by referral to animal hospital and/or Seoul National University Hospital for Animal. After surgical removal and hospital discharge, regular check-up was performed. This included thoracic X-ray and ultrasound of the abdomen and proceeds to CT if necessary. Regular check-up was performed every month after discharge, and if there was no change in the exam results after that, the test was then conducted every 6

months or 1 year. Dogs with malignant CMTs that died of non-tumor related causes or those in which a follow-up was not obtained were excluded from the evaluation of DFS and OS.

1.5. Statistics

The $\chi 2$ test was used to determine the correlation between the TRPM7 expression and the pathological parameters (tumor grade, Ki-67 and tumor size), histological parameters and IHC results. Kaplan-Meier survival curves were plotted and compared using the log–rank test. All the statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA). A $p<0.05$ was considered to be statistically significant.

2. Results

2.1. Dogs

A total of 57 dogs diagnosed with CMTs were included in this study. The signalment data are presented in Table 1. The median age of dogs with benign CMTs was 11.00 years (range: 6–16) and was similar to that of dogs with malignant CMTs (11.94 years; range: 6 – 15). Forty dogs were sexually intact females, while 17 dogs were spayed females. The major breeds were Yorkshire Terrier (n = 17) and Maltese (n = 10). CMT samples were classified into the following four groups: complex adenoma (n=19), simple adenomas (n=8), benign mixed tumors (n = 9), and carcinoma (Grade I, n = 6; Grade II, n = 7 and Grade III, n = 8) (Table 1). Table 2 shows the relationship between the TRPM7 expression and clinical parameters (age, neuter status and breed). The TRPM7 expression was significantly different among breed ($p = 0.003$).

2.2. Immunohistochemistry result of TRPM7 expression in CMTs

The expression of TRPM7 channel in CMT tissues was determined using IHC. IHC staining showed that TRPM7 is diffusely expressed in the areas except the nucleus of neoplastic epithelial cells. In addition, no immunoreactivity was observed in myoepithelial cell of complex adenomas and mesenchymal areas of benign mixed tumors. The cell population was observed in mesenchymal area. TRPM7 expression in these cells was higher than that in the adjacent non-cancerous cells (Figure 1A –

D).

2.3. Correlation between TRPM7 overexpression and diagnosis based on histopathological features, tumor size and Ki- 67

To investigate the role of TRPM7 in CMTs, the relationship between TRPM7 expression and histopathological features was analyzed. Out of 57 CMTs, 36 were benign and 21 were malignant. TRPM7 was overexpressed in 3/36 (8.34%) benign CMTs and 14/21 (66.67%) malignant CMTs. Table 3 shows IHC analysis result and association between TRPM7 expression in benign and malignant CMT tissues. TRPM7 overexpression was observed in simple adenoma (12.5%, n = 8), complex adenoma (5.26%, n = 19) and benign mixed tumor (11.12%, n = 9). In addition, its overexpression was observed in grade I (50%, n = 6), grade II (57%, n = 7) and grade III (85%, n = 8) in malignant tumor. According to histopathological diagnosis, TRPM7 overexpression was not statistically different in benign CMTs ($p = 0.942$) and malignancy grade ($p = 0.137$). Table 4 shows TRPM7 expression was significantly associated with higher Ki-67 ($p = 0.025$) and larger tumor size ($p = 0.016$) in highly malignant tumors. In benign tumors, the correlation between histopathological diagnosis and pathological features was limited as it was difficult to obtain statistical data due to the small number of benign tumors showing overexpression of TRPM7.

2.4. Correlation of TRPM7 overexpression with clinical outcome

The prognostic value of TRPM7 overexpression in malignant CMTs was determined by Kaplan-Meier analysis. We categorized TRPM7 expression as low (0, +1) or high (+2, +3) for survival analysis. All of 21 malignant CMT samples were classified into two TRPM7 groups: high (n = 14) and low (n = 7). Survival curves showed a significant difference between the high and low TRPM7 expression groups; the high TRPM7 expression group was associated with poor DFS (median 13 months vs. 36 months, $p = 0.035$) and shorter OS (median 16 months vs. 42 months, $p = 0.011$) (Figure 2A and 2B). Dogs with high TRPM7 expression had worse prognosis than those with low expression of TRPM7 in CMTs.

3. Discussion

Dysregulation of Ca^{2+} may act more as a "driver" than a "passenger" in carcinogenesis (Cui et al., 2017). Moreover, an increase in Ca^{2+}/ Mg^{2+} ratio is associated with increased risk for postmenopausal breast cancer in human. Increasing number of investigations show that TRPM7 is a valuable diagnostic and prognostic marker of cancer progression related with clinicopathological parameters in human breast cancer (Dhennin-Duthille et al., 2011, Middelbeek et al., 2012, Dhennin-Duthille et al., 2014). Several studies have demonstrated that TRP channels play an important role in diverse cellular functions and are a key factor for tumorigenesis and cancer development (Guilbert et al., 2009, Clark et al., 2006). TRPM7 is a ubiquitously expressed protein and plays a prominent role in early embryogenesis and organogenesis (Jie et al., 2012; Duan et al., 2018). In mammalian cells, TRPM7 channel performs both channel and kinase functions (Krapivinsky et al., 2014). This unique ion channel in combination with an α-kinase is termed as "chanzyme" (Gautier et al., 2016). This "chanzyme" channel is known to transport divalent cations, such as Ca^{2+} and Mg^{2+}. TRPM7 can modulate cell proliferation, migration, adhesion, apoptosis, and necroptosis by facilitating Ca^{2+} influx, whereas Mg^{2+} influx regulates cell proliferation and apoptosis (Dhennin-Duthille et al., 2011). TRPM7 is involved in cell cycle progression, adhesion, survival and migration of cancer cells (Yee et al., 2012). The TRPM7 channel is widely expressed in various organs, including heart, lung, liver, brain and spleen (Nadler et al., 2001). TRPM7 is overexpressed in various types of cancers such as ovarian carcinoma, retinoblastoma, neck and head carcinoma, prostate cancer, lung cancer and pancreatic

adenocarcinoma (Wang et al., 2014a; Jiang et al., 2007; Sun et al., 2013; Gao et al., 2011; Yee et al., 2012a). In particular, TRPM7 is expressed in human breast cancer and normal breast tissues. Furthermore, it has been identified as a breast cancer diagnostic and prognostic marker (Guilbert et al., 2009). Aberrant TRPM7 expression in human breast and pancreatic cancer is closely correlated with clinicopathological parameters, such as tumor malignancy, Ki-67 proliferation index and patient survival time (Zhou et al., 2014). Another study proves that TRPM7 is necessary for pancreatic cancer cell invasion (Yee et al., 2015). Despite abundant knowledge on TRPM7-related carcinogenic pathways in human breast cancer, its role in CMT pathogenesis is still poorly understood. In this study, we show that TRPM7 is overexpressed in highly malignant CMTs. Hence, TRPM7 may represent as an independent prognostic factor for DFS and OS in dogs with CMTs.

In this study, TRPM7 expression was determined in 57 CMTs from dogs. IHC analysis showed that TRPM7 is overexpressed in the cytoplasm of CMTs. This immunostaining reactivity is consistent with previous observations in human breast cancer (Dhennin-Duthille et al., 2011; Guilbert et al., 2009). In contrast to immunoreactivity at the apical membrane of ductal epithelial cells of canine normal mammary gland, TRPM7 was diffusely expressed in the cytoplasm at elevated levels in CMTs. This pattern was also observed in the pancreas wherein TRPM7 was expressed in apical plasma membrane of pancreatic ductal epithelia and in the cytoplasm of pancreatic adenocarcinoma cells (Yee et al., 2011). We further discussed whether other TRP channels show differential immunoreactivity (plasma membrane or cytoplasm) in normal cell and tumor state. TRPM8 protein was localized to the plasma membrane of cells in the normal prostate tissue, whereas its

channel showed severely internalized pattern of TRPM8 in tumor tissues (Bidaux et al., 2007) (Asuthkar et al., 2017). Taken together, these data suggest that IHC staining results of pancreatic and prostate in human normal and cancer cells differ from that of human mammary gland results; however, unlike in human mammary gland tissues, TRPM7 channels are internalized in CMTs, while it is expressed in the apical membrane of ductal epithelial cell of normal mammary gland tissue. Previous studies have demonstrated that TRPM7 mRNA and protein are expressed in canine normal mammary glands. Furthermore, IHC staining of TRPM7 confirmed its localization at the apical membrane of ductal epithelial cells. These results have been reported as the first evidence of the presence and distribution of TRPM7 in canine mammary glands (Lee Sungin, 2020). Despite the vast number of studies on TRPM7 over the past decade, its function and mechanism of action are not fully understood. TRPM7 expression can vary according to the tissue type and its localization and gating in plasma or intracellular membranes (Krapivinsky et al., 2014).

Approximately 30% of CMT samples from both benign and malignant tissues showed moderate (+1) and strong positive (+2) immunoreactivity for TRPM7 expression. TRPM7 expression was higher in malignant CMTs. (14/21, 66.67%) than in benign CMTs (3/36, 8.34%). Moreover, the correlation between TRPM7 overexpression and pathological parameters (proliferative index Ki-67 or tumor size) according to tumor grade was assessed. These parameters are widely known as prognostic factors in canine mammary tumors (Misdorp and Hart, 1976). Our results showed a statistically significant association between TRPM7 overexpression and high Ki-67 and large tumor size, which is consistent with the results of human studies (Guilbert et al., 2009). The positive correlation between tumor progression and

TRPM7 expression may be due to the role of TRPM7 in cancer development. As mentioned previously, TRPM7 plays different roles during cancer progression. TRPM7 is required for cell proliferation and migration as well as epithelial-mesenchymal transition in the early stages and for the regulation of Ca^{2+} and Mg^{2+} homeostasis during cell proliferation, migration, and invasion in the advanced-stage. In addition, aggressive tumors require TRPM7 channel activity and interaction with cytoskeletal proteins (Isabelle Dhennin-Duthille, 2014).

Our findings demonstrated that TRPM7 overexpression is correlated with poor DFS and OS. These results not only suggest a prominent role of TRPM7 in cell cycle regulation and proliferation in CMTs, but also imply a promising application of TRPM7 as a valuable prognostic marker. Additionally, human studies have shown that TRPM7 overexpression in patients with breast, ovarian, and pancreatic cancer is significantly associated with OS and DFS (Middelbeek et al., 2012; Wang et al., 2014b; Rybarczyk et al., 2012).

It should be noted that this study has several limitations. Firstly, the analyzed sample size of benign and malignant CMT is relatively small. Obtaining a larger sample sizes would help to obtain reliable results and also allows multi-perspective analysis. Secondly, this study did not evaluate the relative mRNA and protein level by real-time PCR and western blot. Further investigations are required to address these issues to advance our understanding of TRPM7 function and regulation in health and disease.

The importance of ion channels in various cancers is well known. This knowledge has immensely contributed to the identification of chemotherapeutic agents for several cancers (Cui et al., 2017). Because our previous study showed that

TRPM7 may play a role in the normal physiology and cell functions in the canine mammary gland, further studies on the development of anticancer drugs in dog targeting ion channel signaling proteins in dogs are required. Importantly, a large number of malignant and metastatic CMTs are required to validate the TRPM7 expression as a prognostic factor. Future studies should understand the mechanism of TRPM7 regulation of carcinogenesis. The results of previous and the current study on TRPM7 can help develop novel and effective treatment strategies against CMTs and other malignant tumors. In human studies, TRPM7 finds potential not only as a biomarker in various tumors, but also as a therapeutic target. Ultimately, we need to discuss the potential function of TRPM7 channel-kinase as a biomarker and therapeutic target for achieving the goal of veterinary oncology (Yee, 2017).

4. Conclusion

In this study, we showed that TRPM7 is expressed in the cytoplasm of benign and malignant CMT cells. Furthermore, we demonstrated that TRPM7 expression is positively correlated with prognostic factors such as histological grade, Ki-67 proliferative index and tumor size of in higher grade of malignant CMTs. Our findings demonstrate that high TRPM7 expression is significantly associated with DFS and OS. Thus, TRPM7 expression may serve as a valuable diagnostic and prognostic marker of cancer development with clinicopathological factors in CMTs. However, further studies are required to understand by which TRPM7 overexpression promotes development of CMTs.

Table 1. Comparison of signalment data (age, sex, breed, and histologic diagnosis) of benign and malignant mammary gland tumor in 57 patients.

	Benign Tumors (n = 36)	**Malignant Tumors (n = 21)**
Median age (range)	11.00 (6–16)	11.94 (6–15)
Sex (n)	Female (26) Spayed female (10)	Female (14) Spayed female (7)
Breed (n)	Yorkshire Terrier (14) Maltese (7) Poodle (3) Cocker Spaniel (4) Mixed (2) Schnauzers (2) Chihuahua (1) Miniature Pinscher (1) Boston Terrier (1) Shih-tzu (1)	Maltese (7) Yorkshire Terrier (3) Poodle (3) Shih-tzu (4) Jindo (1) Malinois (1) Cocker Spaniel (1) Dachshund (1)
Histologic diagnosis (n)	Complex adenoma (19) Simple adenoma (8) Benign mixed tumor (9)	Carcinoma Grade I (6) Carcinoma Grade II (7) Carcinoma Grade III (8)

Table 2. Immunohistochemical results of 36 benign and 21 malignant canine mammary tumor (CMTs) from dogs.

Histopathological diagnosis	No. of tumors	TRPM7 overexpression		p
		No.	%	
Benign CMT				
Simple adenoma	8	1	12.5	
Complex adenoma	19	1	5.3	
Benign mixed tumor	9	1	11.1	
Total	36	3	8.34	0.942
Malignant CMT				
Mammary carcinoma, Grade I	6	3	50.0	
Mammary carcinoma, Grade II	7	4	57.0	
Mammary carcinoma, Grade III	8	8	85.0	
Total	21	15	66.7	0.137

The correlation between transient receptor potential melastatin 7 (TRPM7) expression and histopathological diagnosis in 57 CMTs from dogs using $\chi 2$ analysis is shown.

Table 3. Correlation between TRPM7 overexpression and diagnosis based on malignancy grade, tumor size and Ki- 67 expression

Tumor Grade	No. of tumors	Ki-67 ≤15%		Ki-67>15%		χ^2	No. of tumors	Size ≤3 cm		Size >3 cm		χ^2
		No.	%	No.	%			No.	%	No.	%	
Mammary carcinoma, Grade I	3	2	66.7	1	33.3		3	2	66.7	1	33.3	
Mammary carcinoma, Grade II	4	1	25	3	75		4	0	0	4	100	
Mammary carcinoma, Grade III	8	0	0	8	100		8	0	0	8	100	
Total	15	3	66.7	12		0.024	15	2		13		0.017

TRPM7 was overexpressed in 15 out of 57 dogs. TRPM7 overexpression with Ki-67 > 15% and tumor size > 3 cm was observed in 12 and 13 dogs, respectively. $p = 0.005$ was considered statistically significant.

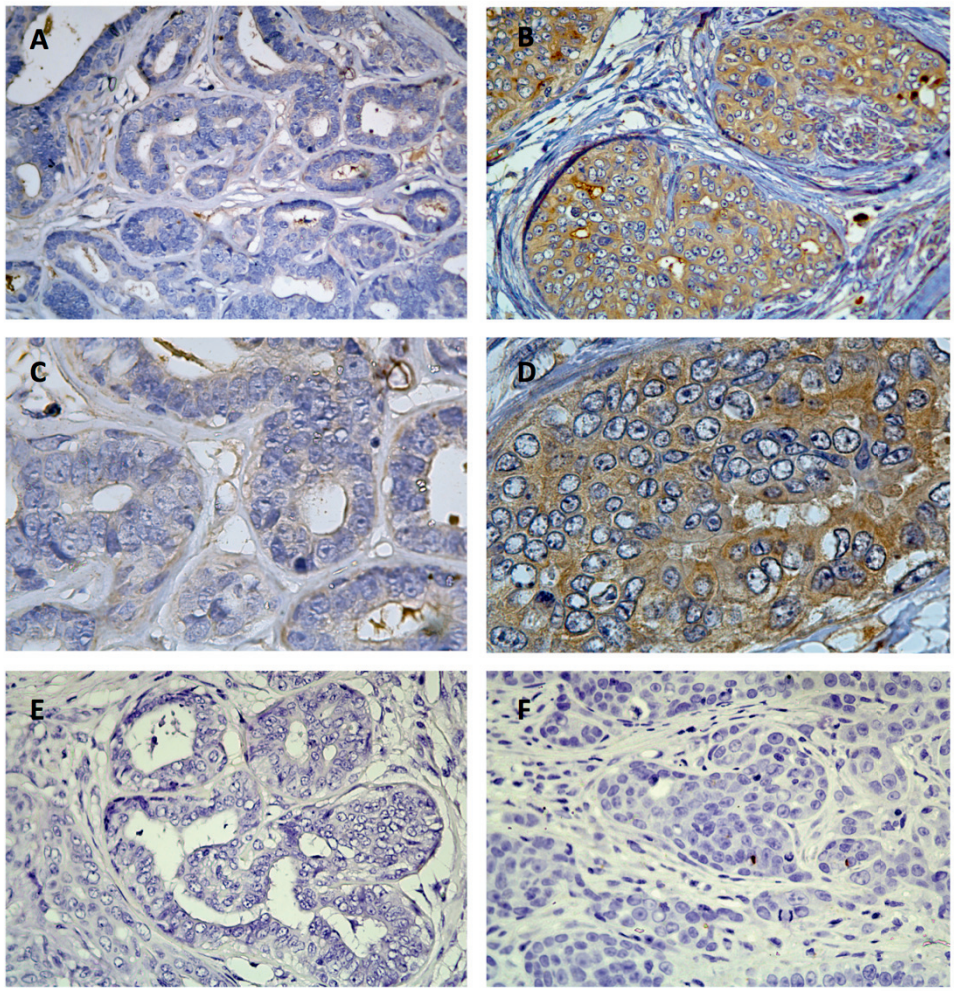

Figure 1. Immunohistochemical staining of TRPM7 in canine mammary gland tissue (CMTs). (A) and (C) Benign CMT (ductal adenoma) with low TRPM7 expression (weak positive). (B) and (D) Malignant CMT (Grade III; solid type) with high TRPM7 expression (strong positive). (E) No specific staining was observed in the negative control samples of benign CMTs. (F) No specific staining was observed in the negative control in malignant CMTs. Sections were counterstained with hematoxylin. (A, B, E, F original magnification ×400; C, D original magnification ×1000). CMT, canine mammary tumor.

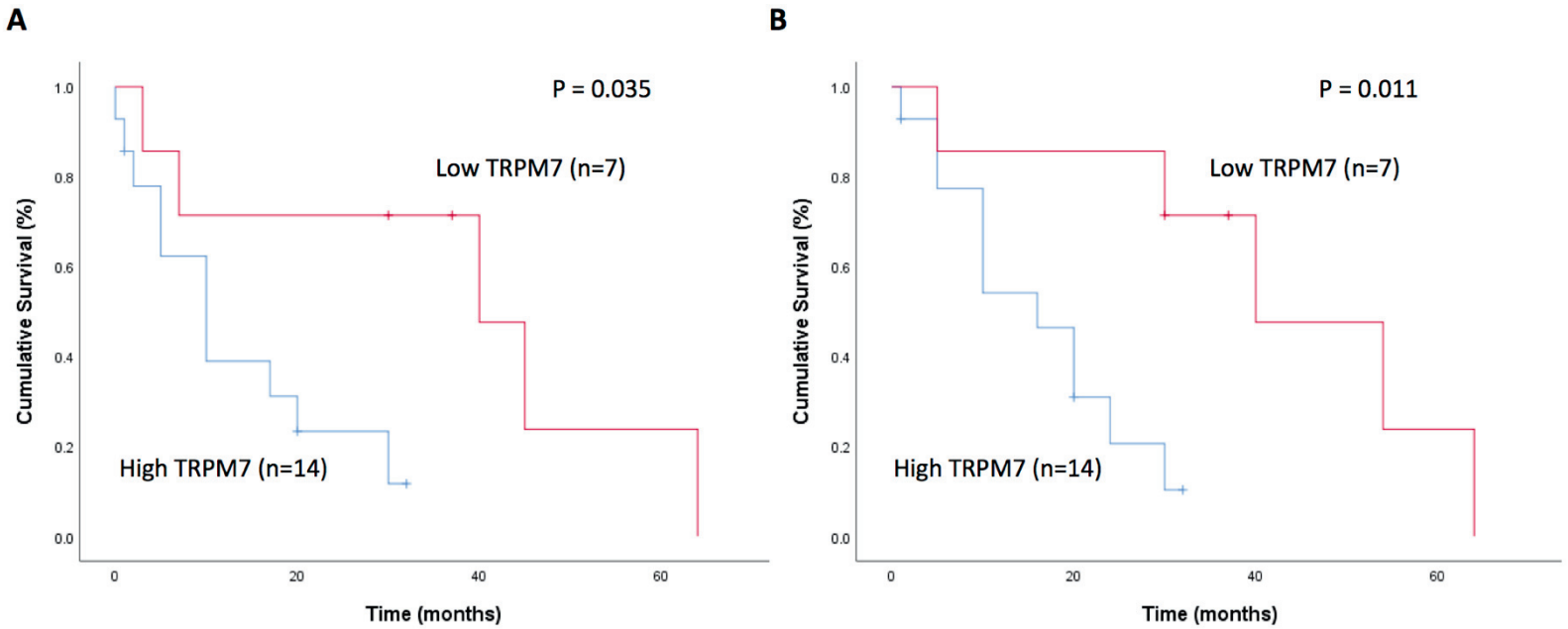

Figure 2. Kaplan-Meier survival curves of 21 dogs with malignant CMTs based on TRPM7 expression status for (A) Disease-free Survival (median: 18 months) and (B) Overall survival (median 22 months).

6. References

Asuthkar S, Demirkhanyan L, Mueting, Sr., et al. High-throughput proteome analysis reveals targeted TRPM8 degradation in prostate cancer. Oncotarget 2017;8:12877-12890.

Bidaux G, Flourakis M, Thebault S, et al. Prostate cell differentiation status determines transient receptor potential melastatin member 8 channel subcellular localization and function. Journal of Clinical Investigation 2007;117:1647-1657.

Bödding M. TRP proteins and cancer. Cellular Signalling 2007;19:617-624.

Chen JP, Luan Y, Yu R, et al. Transient receptor potential (TRP) channels, promising potential diagnostic and therapeutic tools for cancer. BioSci Trends 2014;8:1-10.

Clark K, Langeslag M, Van Leeuwen B, et al. TRPM7, a novel regulator of actomyosin contractility and cell adhesion. EMBO Journal 2006;25:290-301.

Cui C, Merritt R, Fu L, et al. Targeting calcium signaling in cancer therapy. Acta Pharmaceutica Sinica B 2017;7:3-17.

Dhennin-Duthille I, Gautier M, Faouzi M, et al. High Expression of Transient Receptor Potential Channels in Human Breast Cancer Epithelial Cells and Tissues: Correlation with Pathological Parameters. Basel, Switzerland, 2011;813-822.

Dhennin-Duthille I, Gautier M, Korichneva I, et al. TRPM7 involvement in cancer: a potential prognostic factor. Magnesium research 2014;27:103-112.

Dowsett M, Nielsen TO, A'Hern R, et al. Assessment of Ki67 in Breast Cancer: Recommendations from the International Ki67 in Breast Cancer Working Group. Journal of the National Cancer Institute 2011;103:1656-1664.

Duan J, Li Z, Li J, et al. Structure of the mammalian TRPM7, a magnesium channel required during embryonic development. Proceedings of the National Academy of Sciences of the United States of America 2018;115:E8201-E8210.

Gao H, Chen X, Du X, et al. EGF enhances the migration of cancer cells by up-regulation of TRPM7, 2011;559-568.

Gasperetti F, Ferro A, Cuorvo LV, et al. Proliferative activity in human breast cancer: Ki-67 automated evaluation and the influence of different Ki-67 equivalent antibodies. Diagnostic Pathology 2011;6:S7.

Gautier M, Perrière M, Monet M, et al. Recent Advances in Oncogenic Roles of the TRPM7 Chanzyme. Current Medicinal Chemistry 2016;23:4092-4107.

Gkika D, Prevarskaya N. Molecular mechanisms of TRP regulation in tumor growth and metastasis. BBA - Molecular Cell Research 2009;1793:953-958.

Goldschmidt MH, Peña L, Zappulli V. Tumors of the mammary gland. Tumors in domestic animals 2016:723-765.

Guilbert A, Gautier M, Dhennin-Duthille I, et al. Evidence that TRPM7 is required for breast cancer cell proliferation, 2009;C493-C502.

Isabelle Dhennin-Duthille MG, Irina Korichneva, Halima Ouadid-Ahidouch. TRPM7 involvement in cancer: a potential prognostic factor. Magnesium Research 2014;27:103-112.

Jiang J, Li M-H, Inoue K, et al. Transient receptor potential melastatin 7-like current in human head and neck carcinoma cells: role in cell proliferation, 2007;10929.

Jie J, Long-Jun W, Janice J, et al. The channel kinase, TRPM7, is required for early embryonic development. Proceedings of the National Academy of Sciences 2012;109:E225.

Johnston SA. Veterinary surgery : small animal In: Johnston SA,Tobias KM, eds. 2nd ed.. ed. St. Louis, MO: St. Louis, MO : Elsevier, 2018.

Kadthur JC, Rao S, Laxmikanth SM, et al. Prognostic value of Ki 67 proliferation antigen in canine malignant mammary gland tumours, 2011;36-40.

Krapivinsky G, Krapivinsky L, Manasian Y, et al. The TRPM7 Chanzyme Is Cleaved to Release a Chromatin-Modifying Kinase. Cell 2014;157:1061-1072.

Lee S, Lee A, Sim H, Kim G., Kang B and Kim W. The Presence and Distribution of TRPM7 in the Canine Mammary Glands. Animals 2020;10:466.

Lee WJ, Monteith GR, Roberts-Thomson SJ. Calcium transport and signaling in the mammary gland: Targets for breast cancer, 2006;235-255.

Lee WJ, Roberts-Thomson SJ, Holman NA, et al. Expression of plasma membrane calcium pump isoform mRNAs in breast cancer cell lines. Cellular Signalling 2002;14:1015-1022.

Middelbeek J, Kuipers AJ, Henneman L, et al. TRPM7 is required for breast tumor cell metastasis In: Middelbeek J, ed, 2012;4250-4261.

Misdorp W, Hart AAM. Prognostic Factors in Canine Mammary Cancer. Journal of The National Cancer Institue 1976;56:779 -786.

Nadler MJS, Hermosura MC, Inabe K, et al. LTRPC7 is a Mg·ATP-regulated divalent cation channel required for cell viability. Nature 2001;411:590-595.

Nassiri MR, Hosseini SA, Ghovvati S, et al. Evaluation of Estrogen receptor α and ß genes expression in normal and neoplastic mammary gland in dogs by real-time PCR, 2018;140-146.

Ouadid-Ahidouch H, Dhennin-Duthille I, Gautier M, et al. TRP channels: diagnostic markers and therapeutic targets for breast cancer?, 2013;117-124.

Putluri N, Maity S, Kommagani R, et al. Pathway-Centric Integrative Analysis Identifies RRM2 as a Prognostic Marker in Breast Cancer Associated with Poor Survival and Tamoxifen Resistance. Neoplasia 2014;16:390-402.

Rodrigues, T., Sieglitz, F., & Bernardes, G. J. (2016). Natural product modulators of transient receptor potential (TRP) channels as potential anti-cancer agents. Chemical Society Reviews, 45(22), 6130-6137.

Rybarczyk P, Gautier M, Hague F, et al. Transient receptor potential melastatin-related 7 channel is overexpressed in human pancreatic ductal adenocarcinomas and regulates human pancreatic cancer cell migration. International Journal of Cancer 2012;131:E851-E861.

Santos AA, Lopes CC, Ribeiro JR, et al. Identification of prognostic factors in canine mammary malignant tumours: a multivariable survival study. BMC Veterinary Research 2013;9:1.

Sergeev I. Calcium signaling in cancer and vitamin D. J Steroid Biochem Mol Biol 2005;97:145-151.

Singh R, Gupta P, Kloecker GH, et al. Expression and clinical significance of CXCR5/CXCL13 in human non-small cell lung carcinoma.(chemokine receptors)(Report). International Journal of Oncology 2014;45:2232.

Sun Y, Selvaraj S, Varma A, et al. Increase in serum Ca2+/Mg2+ ratio promotes proliferation of prostate cancer cells by activating TRPM7 channels In: Sun Y, ed, 2013;255-263.

VanHouten J. Calcium Sensing by the Mammary Gland. Journal of Mammary Gland Biology and Neoplasia 2005;10:129-139.

Wang J, Liao Q-J, Zhang Y, et al. TRPM7 is required for ovarian cancer cell growth, migration and invasion, 2014;547-553.

Wang J, Xiao L, Luo C-H, et al. Overexpression of TRPM7 is associated with poor prognosis in human ovarian carcinoma. Asian Pac J Cancer Prev 2014;15:3955-3958.

Withrow SJ, Vail DM, Page RL. Withrow & MacEwen's small animal clinical oncology. Small animal clinical oncology. 5th ed. / [edited by] Stephen J. Withrow,

David M. Vail, Rodney 1. Page.. ed. St. Louis, Mo.: St. Louis, Mo. : Elsevier/Saunders, 2013.

Yee N, Kazi A, Yee R. Cellular and Developmental Biology of TRPM7 Channel-Kinase: Implicated Roles in Cancer. Cells 2014;3:751-777.

Yee NS. Role of TRPM7 in cancer: potential as molecular biomarker and therapeutic target. Pharmaceuticals 2017;10:39.

Yee NS, Chan AS, Yee JD, et al. TRPM7 and TRPM8 Ion Channels in Pancreatic Adenocarcinoma: Potential Roles as Cancer Biomarkers and Targets, 2012.

Yee NS, Kazi AA, Li Q, et al. Aberrant over-expression of TRPM7 ion channels in pancreatic cancer: required for cancer cell invasion and implicated in tumor growth and metastasis. Biology open 2015;4:507-514.

Yee NS, Zhou W, Liang IC. Transient receptor potential ion channel Trpm7 regulates exocrine pancreatic epithelial proliferation by Mg^{2+} -sensitive Socs3a signaling in development and cancer. Disease Models & Mechanisms 2011;4:240-254.

Zhou W, Guo S, Xiong Z, et al. Oncogenic role and therapeutic target of transient receptor potential melastatin 7 channel in malignancy. Expert opinion on therapeutic targets 2014;18:1177-1196.

국문 초록

개 유선 종양의 진단 및 예후 마커로서의 TRPM7

지도교수 김 완 희

서울대학교 대학원
수의학과 임상수의학 수의외과학 전공
이 슬 지

Transient receptor melstatin potential 7 (TRMP7)은 단백질 키나제 도메인을 함유하는 칼슘 및 마그네슘 투과성 양이온 채널이다. 이 연구의 목적은 면역 조직 화학 염색법을 사용하여 개의 양성 및 악성 악성 유선 종양 조직에서 TRPM7 발현을 확인하고 Ki-67 증식 인자 및 임상 병리학적 특징과의 상관성을 평가하는 것이다. Ki-67 발현 (≤15%, >15%) 및 종양 크기 (≤3cm, >3cm)를 평가 하였다. 면역화학염색 분석은 TRPM7이 암 상피 세포의 세포질에서 발현됨을 보여 주었다. 또한, TRPM7은 3/36 (8.34%) 양성 유선종양 및 14/21 (66.67%) 악성 유선종양에서 과발현되었다. 또한, TRPM7 발현은 악성도가 높은 종양에서 더 높은 Ki-67 인자 및 더 큰 종양 크기와 유의적으로 관련성이 확인되었다. 생존 곡선 분석은 높은 TRPM7 발현이 불량한 무병 및 전체

생존과 유의하게 관련됨을 나타냈다. 이 연구 결과는 TRPM7 채널은 개 높은 악성도의 유선종양에 과발현이 확인되며 TRPM7 과발현은 임상병리학적 특징과 상관관계가 확인이 되는것을 증명한다. 결론적으로, 높은 TRPM7 과발현은 개 유선종양에서 가치있는 진단 및 예후 바이오마커로서 가능성을 제시하였다.

주요어 : 개, 이온채널, 면역화학염색, 개 유선종양, TRPM7

학 번 : 2016-21780