The expression of tumor endothelial marker 8 in mammary gland tumor, and the effects of endotrophin on neoplastic cells

Summary of Doctoral Thesis

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Tumor endothelial marker 8 (TEM8), which is one of the TEM gene family, is selectively expressed in tumor vascular endothelial cells, and is known as ANTXR1 (Anthrax toxin receptor 1). TEM8 is considered to be involved in angiogenesis and tumor angiogenesis, and TEM8 expression is a positive correlation with poor prognosis. It has been ever reported that there are five splicing variants and three TEM8-isoform (long, medium, short) that are corresponding to each variants. However, there are no reports on TEM8 in canines, and the expression and localization of TEM8 have been unclear.

In this study, we initially investigated the histological localization of TEM8 and expression of TEM8-isoforms in canine normal tissues (Chapter 2), and developmental alterations of TEM8 expression in normal mammary gland (MG) epithelium (Chapter 3) by immunohistochemistry in order to clarify the expression and significance of TEM8 in canine mammary gland tumor (MGT). Thereafter, we investigated the phenotypical characteristics of TEM8 expressing MGT cells in canine MGT cases and canine MGT cell lines by immunohistochemistry and *in vitro* analysis (Chapter 4). Furthermore, we also examined the effects of endotrophin (ETP) on the MGT cells that is type VI collagen (α3 chain)-cleavage product and physiological ligand of TEM8, and the function of HIF-1α on regulating TEM8 expression in MGT cells (Chapter 5).

Chapter 2; The expression and localization of TEM8 in the canine normal tissues and the tissue-specific expression of TEM8-isoform

In this chapter, we initially invested the histological localization of TEM8 in canine normal tissues by immunohistochemistry and RT-PCR analysis as a previous study to clarify TEM8 expression in canine MGT. Furthermore, we

examined the distribution of TEM8-isoform in each tissue by western blot analysis. The results showed that TEM8 was extensively expressed in canine normal tissues including the mammary gland, and the expression of TEM8-isoform in predominant was different in each tissue. This indicated that TEM8 might have a important role for various tissue, moreover, the function of angiogenesis within the tumor and as ANTXR1. It was also revealed that TEM8 expression in MG was localized to mammary epithelial cells and both the membrane-bound type (long, medium isoform), which functions as the receptor, and the soluble type (short isoform), which functions as the soluble factor, was dominantly expressed in MG. Furthermore, almost organ which formed luminal structures expressed the membrane-bound type of TEM8, however, the organs which formed solid structures tended to express the soluble type. The soluble type of TEM8 contained same extracellular domain of membrane-bound type although the real function was not cleared, which indicated that the soluble type might function as a decoy receptor. Therefore, the membrane-bound type functioned as a receptor would be regulated by altering the populations between the soluble and the membrane-bound of TEM8.

Chapter 3; TEM8 expression at the stage of rat mammogenesis

In this chapter, we investigated the developmental alterations of TEM8 expression in rat MG from birth to the stage of lactation by immunohistochemistry in order to clarify the expression change of TEM8 in the mammogenesis. We also examined the relationship between TEM8 expression and the morphogenesis of mammary gland by searching for the expression of Notch-1 and c-MET, which are considered to be involved in the mammogenesis.

TEM8 expression in MG epithelial cells was increased along with the development of luminal structures, and TEM8 expression was related with the expression of Notch-1, which inducing the differentiation of luminal cells, and c-MET, which promoting to form the luminal structures. This indicated that TEM8, which rapidly increased the expression along with the development of MG epithelial cells, would have a important role in the luminal formation and the luminal differentiation of mammary epitheliums.

Chapter 4; TEM8 expression in canine mammary gland tumor and the characteristics of TEM8 (+) neoplastic cells

In this chapter, we investigated the relationship between TEM8 expression and histological features along with mammary tumorigenesis, and analyzed TEM8 expression in 91 cases of canine MGTs and two type of MGT cell lines which derived from luminal and basal cells. Neoplastic cells significantly expressed TEM8 in adenoma - simple, carcinoma - simple and carcinoma - micropappillary invasive, and most of TEM8 (+) neoplastic cells formed luminal structures. However, neoplastic cells, which forming solid structures in carcinoma - solid, significantly decreased TEM8 expression. TEM8 (+) neoplastic cells showed the luminal-like phenotype CK19/p63/ α SMA (+/-/-) but not the basal-like phenotype CK19/p63/αSMA (-/+/-). Notch-1 and c-MET expressions were also confirmed in almost TEM8 (+) neoplastic cells. Furthermore, it was revealed that MGT cell line, which derived from luminal cells, significantly expressed TEM8, Notch-1, c-MET and p-c-MET compared to another MGT cell line, which is derived from basal cells. This indicated that TEM8 expression in canine MGT cells was involved in the differential

regulation of luminal cells via Notch-1 signaling suppressed p63 expression, and in the luminal formation via MET signaling. TEM8 would, therefore, be useful in identified the possible ontogeny of the neoplastic epithelial cells with the luminal differentiation in MGTs.

Chapter 5; Col VI (α3) C5-flagment expression in canine mammary gland tumor, and the effects of Endotrophin on neoplastic cells and the function of HIF-1α on regulating TEM8 expression in neoplastic cells

In this chapter, we conducted immunohistochemistry of Collagen VI (Col VI) α3-chain C5-domain, a source of Endotrophin (ETP), in canine MGTs, and TEM8 (+) neoplastic cells significantly expressed C5-domain. Subsequently, we conducted the ETP-stimulation in canine MGT cell line, which derived from luminal cells. It was revealed that the proliferation of MGT cell line significantly increased by ETP-stimulation. Furthermore, the expression of *CD49b* and *CD44* mRNA significantly increased in MGT cell line, however, *EpCAM* and *CD133* mRNA decreased. It is considered that CD49b is the pure marker of luminal progenitor cells and CD49b+/CD44+ luminal progenitor cells have a high proliferation. Moreover, CD133 would be involved in the luminal forming potency of the luminal progenitor cells, and the bipotencial differentiation of the luminal progenitor cells is effected by decrease in the amount of CD133 expression. EpCAM expression might be increased along with differentiating to mature luminal cells from luminal progenitor cells and/or mammary stem cells. This indicated that ETP/TEM8-signaling would maintain canine MGT cells as luminal progenitor cells which had highly proliferative activity. Recently, it was reported that the luminal progenitor cells had the

bipotential differentiation to luminal and/or basal cells, and there was stem cell in the population of luminal progenitor cells. Therefore, in canine MGTs, the characteristics of bipotential luminal progenitor cells might be maintained by autocrine signals of ETP/TEM8.

It was suggested that Hypoxia inducible factor-1α (HIF-1α), master inducible factor of angiogenesis, might be involved in regulating TEM8 expression due to TEM8 promoting the tumor angiogenesis. Therefore, we investigated the alterations of TEM8 expression by HIF-1α-inducing of canine MGT cell line. HIF-1α-inducing leaded to the decrease of TEM8-long isoform expression and significantly the relative increase of TEM8-short isoform expression. At the same time, the expression of α3 and C5-domain in neoplastic cells tended to decrease by HIF-1α-inducing. This indicated that the function of HIF-1α would enhance the effects of the soluble type by increasing the expression of TEM8-short isoform rather than the signaling pathway between ETP and the membrane-bound type of TEM8 in neoplastic cells.

In this study, we clarified the expression and significance of TEM8 in canine MGTs by immunohistochemistry and molecular biological analysis. In MGTs, TEM8 expression was detected in luminal-like neoplastic cells, and had a relationship between Notch-1 and c-MET expression. This indicated that TEM8 would be involved in the differentiation to luminal cells and the luminal forming potency. Furthermore, TEM8 (+) MGT neoplastic cells, which co-expressed ETP, would have highly proliferative activity and maintain the characteristic of the biphasic luminal progenitor cells, which could differentiate to luminal and/or basal cells. The regulation of TEM8 and C5-domain

expression in the neoplastic cells via HIF-1α-inducing might be also involved in the complex tumor morphological changes. It was considered that several factors were interacted with the morphological changes in various neoplastic cells and tumors. This study indicated that TEM8 expression and the effects of ETP would be important prognostic marker in presuming the biological and clinical behaviors of MGT neoplastic cells. Furthermore, it would be expected that more detailed expression and functional analysis of TEM8-variants were connected for the comprehensive elucidation of tumor morphological functions.