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Increased Expression of MET and RON Receptor Tyrosine Kinases in Canine Cutaneous Melanotic Tumor

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Abstract : Aberrant translocation of β-catenin can be induced by the dissociation of cadherin-catenin complex, which is mediated by the activation of receptor tyrosine kinases (RTKs). We examined the expression levels of MET/RON RTKs in tissue samples of canine cutaneous melanotic tumor. The activation of MET/RON RTKs was observed in 28% of the examined samples. Our results indicate the possibility that the activated MET/RON RTKs are implicated in the dissociation of cadherin-catenin complex in canine cutaneous melanotic tumor.

Key words **:** canine melanotic tumor, MET, RON, RTKs, Wnt/β-catenin signaling pathway.

Introduction

Protein tyrosine kinases (PTKs), a large and diverse class of cell surface receptors, regulate a variety of critical processes such as cellular growth and differentiation (17). Phosphorylation is prerequisite for the activation of PTKs. PTKs are classified into 2 groups on the basis of their target phosphorylation site: tyrosine kinases (TKs) and serine/threonine kinases (13). Receptor tyrosine kinases (RTKs) are the TKs on the cell surface (13). Of the 90 identified tyrosine kinases, 58 are known to be RTKs (17).

The MET RTK family contains 3 members: MET (3), RON (18), and c-Sea (11). The c-Sea is considered as a chicken RON orthologue (11). MET expression is induced in response to tissue damage or regeneration such as organ injury or liver generation, thus it is considered as an important mediator in the wound healing and tissue repair (2). RON is associated with tissue regeneration and development besides mitogenesis and motogenesis in epithelial cells in a manner similar to MET (20). Increased expression of the MET and RON also has been reported in several cancers in humans (6-8,14).

In our previous study, we observed that all the examined tissue samples of canine cutaneous melanotic tumor (CCM) (n = 18) showed substantial expression of *ctnnb1* gene encoding β-catenin and intracellular accumulation of β-catenin (Veterinary pathology, in press). To investigate the cause of its intracellular accumulation, we further investigated the expression levels of MET and RON RTKs in the CCM tissues.

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Materials and Methods

Melanoma Patient Information

This study was performed on the patients that were referred to Veterinary Teaching Hospitals in South Korea. The patients were diagnosed histopathologically as CCM. The diagnosis between melanocytoma and melanoma was made from anisocytosis, anisokaryosis, invasiveness, and mitotic rate. The mean age of all patients with CCM was 9.4 ± 3.4 years old (range: 2-16). The mean age of all patients with benign tumor (n = 16) was 8.9 ± 3.1 years old (range: 2-14). Ten dogs were male; 8 were intact and 2 were neutered. Six dogs were intact females. One patient with malignant tumor was a 12-year old intact male and the other patient with malignant tumor was a 16-year old intact female.

Semiquantitative RT-PCR of MET and RON RTKs

Total RNA was extracted from normal melanocytes (n = 7) and formalin-fixed paraffin-embedded (FFPE) CCM tissues (n = 18). Normal melanocytes were obtained from seven different normal skin tissues of beagle dogs using a laser capture microdissection (LCM) system (MDS Analytical Technologies, Toronto, Canada). For total RNA extraction, TRIzol® Reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) was used for normal melanocytes and PureLink™ FFPE Total RNA Isolation Kit (Invitrogen) was used for FFPE tissues consisting of tumor cells only. In FFPE tissues consisting of tumor cells with surrounding normal tissues, the area of the tumor was resected using the LCM system, and then the Arcturus® Paradise Extraction and Isolation Components Kit (MDS Analytical Technologies) was used. The concentration of total RNA extracted was determined by measuring the absorbance at 260 nm.

Total RNA (0.5 μg) was reverse transcribed into first-strand cDNA using random hexamer (Invitrogen) and Superscript™ First-strand Synthesis System of RT-PCR Kit (Invitrogen). The primer for MET and RON RTKs were designed based on the GenBank database (MET, NM_001002963; RON, AY646195). To determine the conditions for logarithmic phase PCR amplification for target mRNA, cDNAs made by 0.5 μg of total RNAs were amplified using different numbers of cycles. The chloramphenicol acetyltransferase (CAT) gene was used to rule out the possibility of RNA degradation. A linear relationship between PCR products and amplification cycles was observed for target mRNAs. MET and RON genes were quantified using 30 and 25 cycles, respectively. The template cDNA (1 μl) was amplified in triplicate by PCR in a volume of 50 μl containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM $MgCl_2$, dNTP (0.2 mM each) and 0.5 unit of Taq polymerase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The sequences of the primers are shown in Table 1.

PCR products were fractionated on a 2% agarose gel, stained with ethidium bromide and photographed under UV-illumination. The intensity of ethidium bromide-stained PCR bands was semiquantitatively analyzed using a SigmaScan Pro v.6.0.0 (SPSS, Chicago, IL, USA).

Statistical Analyses

All data were analyzed by Student t-test. All statistical analyses were performed with SPSS for Window edition. $P < 0.05$ was considered statistically significant.

Results and Discussion

β-catenin is a mediator of Wnt/β-catenin signaling pathway (12). β-catenin binds to the cytoplasmic domain of cadherin and along with α-catenin establishes a link between cadherin and the actin cytoskeleton (5). In the absence of Wnt signals, a complex of the adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β), and axin protein degrades released β-catenin (12). Danilkovitch et al. reported that activated MET/RON RTKs induce the disruption of cadherin-catenin complex, followed by the dissociation of β-catenin from the cadherin-catenin complex to the cytoplasm in normal canine kidney cells (4). Previously, we have reported a partial loss of membrane β-catenin and its intracellular accumulation in >90% tumor cells in all the FFPE CCM tissues examined (Veterinary pathology, in press). Considering the role of MET/RON RTKs on cadherin-catenin complex, we supposed that the overexpression of these RTKs is related to the alteration of β-catenin expression on cell membrane. Thus, we examined the expression of MET/RON RTKs in the CCM tissues. Consequently, we found that MET and RON were each significantly expressed in 28% (5/18) of the tissues whereas the normal melanocytes barely expressed MET and RON RTKs ($p < 0.05$) (Fig 1). Two benign tissues (1 and 4) expressed both the RTKs. MET and RON were each expressed in 29% (3/11) of male dogs and 27% (2/7) of female dogs. MET or RON RTKs were not expressed in malignant CCM tissues. Microphthallmia transcription factor (MITF) is strongly expressed in canine melanoma (9). Increased expression of MITF expression may lead to increased expression of MET either by a HGF mediated pathway or by a direct contact (1,15). RON shares structural similarities with MET and transmits the signal of a ligand called hepatocyte growth factor-like (HLP) that has the same overall structure as HGF (10). These results suggest that MITF-mediated pathway could

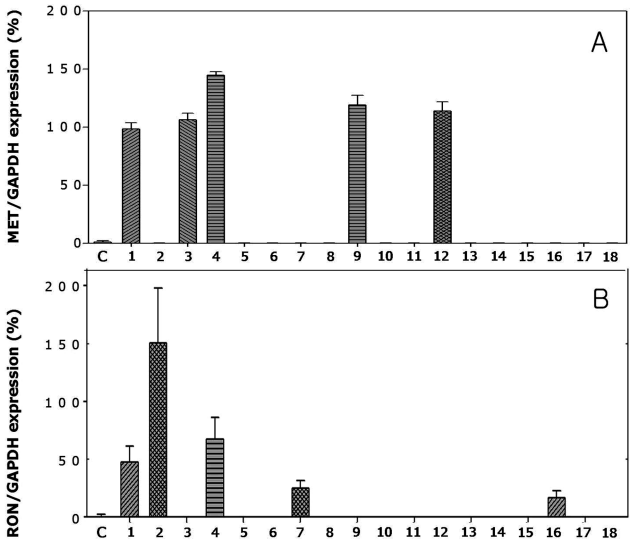

Fig 1. The expression of MET (A) and RON (B) receptor tyrosine kinases (RTKs) in 18 canine cutaneous melanoma tissues. The experiments were performed in triplicate and the data were compared with the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data represents the means ± standard deviation (SD). C, control; No. 1-16, benign melanoma tissues; No. 17-18, malignant melanoma tissues.

Table 1. Oligosequences of the primer pairs used in this study

Name		Sequence (5' to 3')	Annealing temperature (°C)
GAPDH	sense	GCCCT CAATG ACCAC TTTGT	60
	antisense	TCCTT GGAGG CCATG TAGAC	
MET	sense	GGACT TTTCC TGTGG CTGAA	55
	antisense	CAAGC CTATC CAAAT GAGGAG	
RON	sense	AAATG GATGG CACTG GAGAG	53
	antisense	CCATA GCAAC ACACC AAACG	

induce both expressions of MET/RON RTKs whether HGF is involved. However, the low rate of RTKs' expression suggests that another cause that is widely involved in the intracellular accumulation of β-catenin exist and that its alteration may not be related to its histopathological classification. The cause of increased RON expression alone remains unknown.

Cadherins are a family of cell adhesion receptors that are crucial for the mutual association of vertebrate cells (21). While E-cadherin is critical in the formation and maintenance of epithelial structures, N-cadherin is preferentially expressed in migratory cells and in the cells of connective tissue of humans. E-cadherin is widely expressed in canine mammary epithelial cells and partial loss of its expression has been reported in tubular and papillary mammary adenocarcinoma (16). Because β-catenin binds to the cadherin molecule on cell membrane, the loss of cadherin molecule can induce the intracellular accumulation of β-catenin (19). In future, we attempt to examine the cadherin type of the epidermis and to compare the expression levels of cadherin and β-catenin on the cell membrane of FFPE CCM tissues.

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개 피부 흑색종의 MET/RON Receptor Tyrosine Kinases 발현 평가

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요 약 : Cadherin-catenin 복합체의 파괴는 β-catenin 단백질의 발현 위치 이상(세포내 이동)을 유발하며, 일부 receptor tyrosine kinase (RTK)의 활성화가 cadherin-catenin 복합체의 파괴를 유발할 수 있다. 본 연구에서는 β-catenin의 발현 위치 이상을 나타낸 개 피부 흑색종 조직 18개에서 MET/RON RTKs의 발현양을 평가하였다. 실험 결과 총 28%의 종양조직에서 MET/RON RTKs의 발현증가가 관찰되었다. 이 결과는 개 피부 흑색종에서 MET/RON RTKs의 발현증가가 β-catenin의 위치이상에 부분적으로 관여할 가능성을 제시한다.

주요어 : 개 흑색종, MET, RON, RTKs, Wnt/β-catenin signaling pathway.