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# 犬の肝幹細胞に関する研究「犬c-Met/HGFレセプターcDNAのクローニングとその組織分布」

*Studies on canine hepatic stem cell*
*"Molecular cloning of the canine c-Met/HGF receptor and its expression in canine tissues"*

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**Abstract.** The c-Met proto-oncogene is the receptor for hepatocyte growth factor (HGF), which is a member of the tyrosine kinase family. Activation of the HGF/c-Met signal pathway leads to cell proliferation, motility, and morphogenesis. In human and mouse, c-Met is detected in various epithelial and endothelial cells and numerous studies have indicated it to be an important component of cellular function.

In this study, the complete sequence of complementary DNA (cDNA) of canine c-Met was cloned, and its distribution was determined in tissues. The canine c-Met cDNA clone had an open reading frame of 4419 bp which encoded a putative polypeptide of 1383 amino acids. Furthermore, it was indicated that c-Met was expressed in a variety of canine tissues including peripheral blood mononuclear cells (PBMC), bone marrow, liver, kidney, lung, stomach, uterus, testis, thymus, lymph node, small intestine, colon, adrenal grand, thyroid gland, heart, muscle, skin, pancreas, ovary, prostate, spleen, fat, cerebrum and cerebellum. The c-Met m-RNA was overexpressed in the bone marrow cells from acute myelocytic leukemia (1/1), chronic myelocytic leukemia (2/2) and myelodysplastic syndrome (1/3), however, the expression of m-RNA in the bone marrow of chronic lymphocytic leukemia and plasmacytoma was normal bone marrow level.

## INTRODUCTION

Hepatocyte growth factor (HGF) affects a variety of physiological functions. In human, the function of HGF is diverse and affects a number of cellular functions including proliferation, motility and morphogenesis. The c-Met proto oncogene has been identified as the receptor for HGF [1)] and is a member of the cell surface receptor tyrosine kinase family. C-Met is a heterodimeric protein composed of an extracellular $\alpha$-chain and a $\beta$-chain which

spans the plasma membrane and includes an extracellular, a transmembrane and a cytoplasmic domain [2]. In a variety of species, including human, murine, avian and amphibian species, the cDNA has been cloned. The latter has allowed study of the patterns of expression in various tissues. The expression profiles of c-Met mRNA in the various tissues and during various physiological states suggests that HGF has a multiplicity of functions [3-6]. However, the cloning and tissue distribution of canine c-Met has not been reported. Thus, it is not clear if the mRNA expression profiles in the canine are in accord with other species.. In this report, we describe the cloning of the canine c-Met and its tissue distribution in normal or tumor condition.

## MATERIALS AND METHODS

Total RNA was extracted using RNA isolation reagent (Sepagene RV-R, Sanko Jyunyaku, Japan). Total RNA (1 $\mu$g) of liver was denatured for 10 min with oligo dT-primers and reverse-transcribed with 200 units of SuperScript III (Invitrogen) in a final reaction volume of 20 $\mu$l. Based on the sequences of human, rat and mouse[7-9] cDNA , primers were designed. Primer sequences are indicated in Table 1. Partial canine c-Met clones were obtained by PCR amplification between primers F2 (sense primer) and R2 (antisense primer), and between F8 (sense primer) to R8 (antisense primer). Primers (R2, R1 and F8) were used to clone the 5' and 3' end of mRNA by RACE. The 5' region was cloned using R2 and R1, whereas the 3' region was cloned using F8. After sequencing of RACE products, new primers (F12 and R 3) were designed and used in PCR. The amplification profile consisted of 2 min of denaturation at 94°C for the first cycle and 30 sec per cycle thereafter, 30 sec annealing at 55°C to 60°C, and 1 min extension at 72°C for the first 34 cycles and 10 min

Table 1 Sense and antisense primers for amplification of canine c-Met cDNA

| Primer | Primer sequences | Purpose |
|---|---|---|
| F1 | ctggtatggttctttcagtt | * |
| R1 | tcagcagtatgattgtggggaa | Nested primer for 5'RACE |
| F2 | ggagcaatggggagtgtaaaga | * |
| R2 | tgtgaaaagtctgagcatctag | For amplifying 5'cDNA end |
| F3 | tgtccacgcctttgaaagca | * |
| R3 | ggacgtcccaagattagcaa | * |
| F4 | cagttcaaccaagtcctctt | * |
| R4 | gctctcacttaaggtcaagg | * |
| F5 | tggctgggacttcggattca | * |
| R5 | tcagttgcagtggcttgagct | * |
| F6 | gtggcaccttgctcacttta | * |
| R6 | tgttgcagtgaaggagtcgt | * |
| F7 | ggatggtaatagatgtccat | * |
| R7 | agtagctcggtagtctacag | * |
| F8 | gagctaaatatagagtggaagc | For amplifying 3'cDNA end |
| R8 | aatgccctcttcctatgacttc | * |
| F9 | cacattgacctcagtgctct | * |
| R9 | catgtctctggcaagaccaa | * |
| F10 | ctggtggtcctaccatacat | * |
| R10 | agagaagggtatggagcaac | * |
| F11 | aaaatgctggcaccctagag | * |
| R11 | tgtgcggttgctgttggtca | * |
| F12 | cggaattcgaagcttctggtatggttctttcagtt | For amplifying complete length of cDNA |
| R12 | cggaattcgaagctttgtgcggttgctgttggtca | For amplifying complete length of cDNA |

* indicates that the primer was used for amplifying particular sequences of canine c-Met

extension on the final cycle. The amplified PCR products were electrophoresed on 2% agarose gel, excised, purified and ligated into the pCR2.1-TOPO plasmid vector (InVitrogen Life Technology, U.S.A.). DNA sequencing was performed on plasmids using dye-terminator chemistry and the Applied Biosystem Model 310 sequencer by the dideoxy-mediated chain-termination method[10]. Nucleotide sequence analysis was performed with Edit View ABI automated DNA sequence viewer (Perkin Elmer, U.S.A.). We next examined the expression of c-Met in normal canine tissues by RT-PCR. Total RNAs were extracted from peripheral blood mononuclear cells (PBMC), bone marrow, liver, kidney, lung, stomach, uterus, testis, thymus, lymph node, small intestine, colon, adrenal grand, thyroid gland, heart, muscle, skin, pancreas, ovary, prostate, spleen, fat, cerebrum and cerebellum. The c-Met mRNA was detected by RT-PCR using primers F2 and R2 as described above. As the internal control, canine $\beta$-actin mRNA was also amplified by RT-PCR from the same tissues. Bone marrow cells from dogs of one acute myelocytic leukemia (AML), two chronic myelocytic leukemia (CML) and three myelodysplastic syndrome (MDS), three chronic lymphocytic leukemia (CLL), a plasmacytoma (PCT) and a clinically healthy dog were examined expression of c-met mRNA by real- time PCR. Total RNA was extracted using RNA isolation reagent (Sepagene RV-R, Sanko Junyaku, Japan). First-strand cDNA was made from 1 $\mu$g total RNA with specific primer using reverse transcriptase (AMV RT-PCR kit, Takara, Japan) Real time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Forster City, CA, U.S.A.). Based on the TaqMan probe and prime pairs of canine c-Met and canine beta-actin were designed by Primer Express version 1.5 (Applied Biosystems, Forster City, CA, U.S.A.). The c-Met Taqman probe was 5'CAGATTGTTCCCCATGTCAGGACTGCAG3'. The canine c-Met forward primer sequence was 5'GGCCCGTGCTGGAACAC3',and its reverse primer sequence was 5'CACCACCTGATAAATTGGCTTTG3'. Canine beta-actin probe was 5'ATCCTGACCCTGAAGTACCCCATTGAGC3'. Canine beta-actin forward primer was 5'GATGAGGCCCAGAGCAAGAG3' and reverse primer was 5'TCGTCCCAGTTGGTGACGAT3'. The PCR reaction mixture was consisted of distilled water, Taqman probes and primers of c-Met or canine beta-actin, and Taqman Universal Master Mix (Applied Biosystems, Forster City, CA, U.S.A.), and cDNA from each bone marrow sample. The amplification profile was consisted of 2minutes at 50 ℃ for uracil-N-glycosylase incubation, 10 minutes at 95 ℃ for activation of AmpliTaq gold DNA polymerase, and 40 cycles of 95 ℃ for 15 seconds and 60 ℃ for one minutes. The amplification plots of c-Met and canine beta-actin in each sample was analyzed on the point of threshold cycle number and amplification curve to obtain stable quantitative results for the experiments afterwards.

## RESULTS AND DISCUSSION

The sequence obtained (Fig. 1) spanned 4419bp and contained an open reading flame encoding a protein of 1383 amino acids composed of a putative signal peptide of 24 residues and a mature protein of 1358 amino acids. The 5'-untranslated region comprised 59 nucleotides and the 3'-untranslated region was 163 nucleotides which started at the first in-frame stop codon at position 4147. The presence of the sequence within the 5'-UTR (ACCATGA) suggests the translation starting codon as revealed by similarity to the consensus sequence suggested by Kozak[11]. Canine c-Met cDNA shows high identify with human (89%), mouse (85%), rat (87%), chicken (68%), and Xenopus (80%) cDNAs. Comparison of the deduced amino acid sequence of canine c-Met with other known c-Mets revealed that there are several conserved domains. Canine c-Met is 89, 89, 88, 73 and 63% homologous to human, mouse, rat, chicken and xenopus, respectively.

The intramolecular hydrophobic region at position 929-953, is likely to be the transmembrane spanning domain. The tyrosine kinase domain, which is predicted by intracellular regions at amino acid 1079-1338, showed substantial identity among human (93%), mouse (89%), rat (88%), and xenopus (91%). Futhermore, the clone had the expected similarity to highly conserved features of the

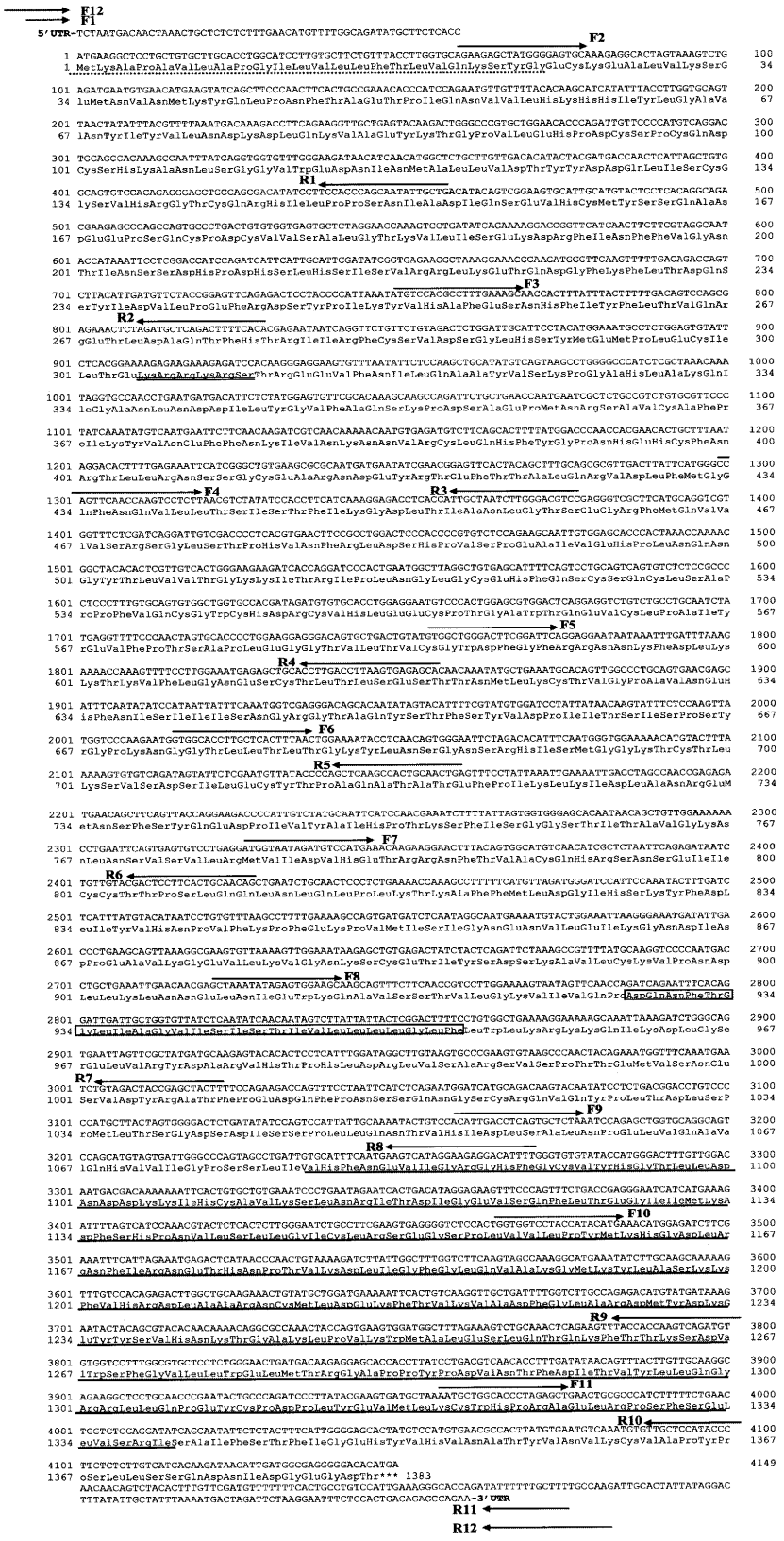


Fig 1. Nucleotide sequence and predicted amino acid sequence of canine c-Met cDNA(Genbank accession NO. AB118945). Nucleotides are numbered beginning with the first methionine of the open reading frame. The predicted signal sequence, the putative cleavage site, the transmembrane domain, and tyrosine kinase domain were indicated by broken line, double underline, box, unbroken under line, respectively. The position of primers used for RT-PCR of canine c-Met was labeled with arrows together with their names.

endoproteolytic protein[12] and contained the consensus sequence (R-X-R/K-R) for the endoprotease furin at position 305-308. The presence of the consensus sequence indicates that canine c-Met is most likely a heterodimeric molecule consisting of $\alpha$ and $\beta$ chains.

The sequence cloned in this study shows highest identity compared to human cDNA. Canine c-Met cDNA, however, lacks the 54 bp predicted to encode 18 amino acids in the extracellular domain compared to that of human. This difference may be explained by alternative splicing, because a similar isoform lacking the 54 bp had been detected in human. Since the latter isoform is dominantly expressed in several cell types of humans[13, 14], these results may indicate that the form cloned in this study is the major form in canine.

In the studies for c-Met mRNA distribution on normal tissue, the predicted 519 bp PCR product was clearly amplified from all canine normal tissues examined in this study (only partially showed in Fig.2). These results are in accord with previous studies in human and mouse[3, 6].

The c-met mRNA was overexpressed in the bone marrow cell of 1 to one AML, 2 to two CML and 1 to three MDS patients. However, expression of the mRNA in CLL and PCT was normal bone marrow level (data not shown). In this study, c-Met m-RNA was overexpressed in myelocytic leukemia especially in granulocyte. In human medicine, HGF was suggested to affect proliferation and migration of myelocytic leukemic cells [15], this phenomenon might be related with c-Met expression. Recently, c-Met proto-oncogene has been also suggested

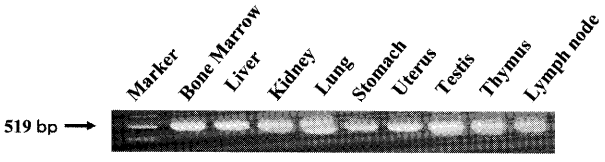


Fig 2. Expression of c-Met in canine tissues were determined by RT-PCR amplification. The 519bp DNA fragment was detected and confirmed expressions of c-Met in these tissues. c-Met was also detected in other tissues including PBMC, small intestine, colon, adrenal grand, thyroid gland, heart, muscle, skin, pancreas, ovary, prostate, spleen, fat, cerebrum, and cerebellum. Canine $\beta$- actin was used as a control. (data not shown.)

to involvement in liver invasion in adult T-cell leukemia[16]. There are some reports concerning canine HGF/c-Met kinetics in both experimental and clinical science. Canine kidney cells and thyroid epithelial cells revealed response to HGF[17, 18]. In addition, transfection of HGF cDNA improved angiogenesis and function of chronic ischemic myocardium in canine heart[19] and overexpression of c-Met is found in canine osteosarcoma[20]. These results suggested that mechanism of HGF/c-Met kinetics might be associated with proliferation and differentiation in various tissues. However, although c-Met mRNA expression has been detected in the various tissues, HGF does not have a stimulatory effect in all tissues tested. The function of the HGF/c-Met signal transduction pathway may be limited to specific or injured tissues[21]. These specific effects may be regulated by other factors. Thus, further analysis of the HGF/c-Met axis and the involvement of other modifying factors is necessary.

In conclusion, cloning of the complete canine c-Met is invaluable for the further analysis of the physiological effects of HGF/c-Met and its involvement in embryogenesis, tissue regeneration and oncogenesis.

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## 和文要約

c-Met proto oncogeneは肝細胞増殖因子(Hepatocyte growth factor: HGF)のレセプターであり，ヒト，マウスではあらゆる上皮，内皮系細胞での発現が認められている。HGF/c-Metシグナル伝達は，細胞の増殖，運動および形態形成などを誘導し，その機能的重要性が示唆されるが，本研究ではイヌのc-Met完全長cDNAのクローニングおよび組織分布の検討を行った。イヌc-Met cDNAのOpen reading frameは4419塩基，1383個のアミノ酸で構成され，さらにmRNAは，末梢血単核球，骨髄，肝臓，腎臓，肺，胃，子宮，精巣，胸腺，リンパ節，小腸，大腸，副腎，甲状腺心臓，筋肉，皮膚，膵臓，卵巣，前立腺，脾臓，脂肪，大脳，小脳組織での発現が確認された。一方，急性骨髄性白血病（1/1），慢性骨髄性

白血病（2/2），骨髄異形生症候群（1/3）の犬の骨髄でC-Met mRNAの過剰な発現が認められたのに対しリンパ球性白血病および形質細胞腫の犬の骨髄での発現は正常組織と同程度であった。

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