

## **Molecular cloning of the cDNA of canine homeodomain-interacting protein kinase 2**

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The research of p53 is being conducted to find the mechanisms of tumorigenesis and to treat various cancers. Homeodomain-interacting protein kinase2 (HIPK2) is an important factor to regulate p53 and to increase the stability of p53. Activation of HIPK2 leads to the selective phosphorylation of p53, resulting in growth arrest and the enhancement of apoptosis. In this study, the canine HIPK2 cDNA fragments were obtained, and their overlapping regions were aligned to give a total sequence of 3489 bp. The canine HIPK2 cDNA (GenBank accession number; AY800385) shares 93% and 90% sequence identity with those of human and mouse HIPK2, respectively. The canine HIPK2 cDNA contains an open reading frame encoding 1163 amino acid residues and the predicted amino acid sequence has 98% and 96% identity with those of human and mouse, respectively. The deduced amino acid sequence of canine HIPK2 has also all domains' sites compared with human and mouse HIPK2. Therefore, these structural similarities suggested that the canine HIPK2 shares the basic biological functions that HIPK2 exhibit in other species.

**Key words:** cloning, dog, HIPK2, p53 regulation

### **Introduction**

Homeodomain-interacting protein kinases (HIPKs) constitute a novel family of nuclear protein kinases. Three members of this family, HIPK1, HIPK2 and HIPK3 have been isolated in human and mouse so far but none of those was isolated in dogs. HIPK2 has been described as a homeodomain-interacting protein kinase, which acts as a co-repressor for homeodomain transcription factors [10]. HIPK2 colocalizes with p53 in nuclear bodies and phosphorylates p53.

The tumor suppressor protein p53 is one of the most

important regulators of cellular growth functions, such as cell cycle arrest, DNA repair, and apoptosis, and is mutated in about 50% of all human tumors [8]. The p53 is important in the cellular response to cellular stresses, UV,  $\gamma$ -ray, and toxins [2,4,11,16]. Under normal conditions, p53 is a short-lived protein that is highly regulated and maintained at low or undetectable levels [11]. However, after stresses, the activation of p53 coordinates a change in the balance of gene expression leading to growth arrest, DNA repair or apoptosis, and these actions prevent the proliferation of genetically damaged cells. It involves several mechanisms including post-translational modifications such as phosphorylation and acetylation of specific residues in the amino-terminal and carboxy-terminal domains [16,18]. In addition to post-translational modifications, protein-protein interactions and subcellular relocation also have a role in the activation of p53 [5,17]. The activation of p53 leads to the transcription of several genes whose products trigger different biological outcomes [6].

Activation of HIPK2 leads to the selective phosphorylation of p53 at Ser46, facilitating CBP-mediated acetylation of p53 at Lys382 and promoting p53-dependent gene expression [7]. The HIPK2 enhances the expression of p53 target genes, resulting in growth arrest and the enhancement of apoptosis [3]. Overexpression of HIPK2 leads to an increase of p53 protein expression or stability [19].

The research of p53 is being conducted to find the mechanisms of tumorigenesis and to treat various cancers. Thus, recently the researches are being conducted actively about the structure, function of HIPK2, and the relationship between HIPK2 and p53. In dogs, the gene therapy with p53 in cancer patients is in experimental stage.

The study about the nucleotide sequence of canine HIPK2 was performed for the development of cancer therapy because the attack rate of cancer has been increased depending on longevity of pets in veterinary field.

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DOG	1	ATGCCCTCAGATGTGCAAGTCTTCTCCCTCACACCTTCAATCAAGTGCCTTCTGTAGT	GTGAAGAACTGAAAGTAGAGCGAGTTCCACCTGGGACATGACTGGGTACGGGTCCAC
Human	1	.....T.....	.....A.....
Mouse	1	.....T.....	.....A.....
DOG	121	AGCAAGTGTATAGCCAGAGCAAGTACCACTTCTCAGCCAGCCACCACAACCGTC	AGCACCTCCTTGCCGATCCCAAAACCAAGCCTACCTTACGAGCAGACCATCATCTTCCCA
Human	121	.....A.C..C.TG..G.....	.....G.....
Mouse	121	.....C.....A.....T.....	.....A.....C.....
DOG	241	GGAAAGCACCAGGACATAGTTGTAAATCAGCAAGTAGCACTTCTGTCACTGGGCAAGTC	CTGGCGGACCACATAACCTAATGCGTCGAAGCACTGTGAGCCTTCTTGATACCTACCAA
Human	241	.....C..G..C..C.....C.....	.....C.....
Mouse	241	.....T..T..A.....	.....C.....C.....
DOG	361	AAATGTGGACTCAAGCGTAAGAGTGAGGAGATTGAGAACACAGCAGCGTCAGATCATT	GAAGAGCATCCACCCATGATTGAGAATATGCAAGTGGGGCCACCGTAGCCACTGCCACC
Human	361	.....C.....C.....	.....C.....T..C.....
Mouse	361	.....C.....	.....C.....T.....
DOG	481	ACATCGACTGCCACCTCCAAAACAGTGGCTCTAACAGCAAGGTGATTACCAGCTGGTT	CAGCATGAGGTGCTGTGCTCCATGACCAACACTTACGAAGTTTGAATTCCTGGGCCGA
Human	481	..G..T.....C.....C.....G..C..T.....G.....	.....C.....G..C.....G..T.....
Mouse	481	.....C.....T.....G..C..T.....G.....	.....A..A.....G..T.....G..
DOG	601	GGGACGTTTGGGCAAGTGGTCAAGTGTGGAAACGGGACCAACGAAATCGTGGCCATC	AAGATCCTGAAGAACCCCGTCATATGCCCGCAAGGTCAGATGAGGTGAGCATCCTG
Human	601	.....T..G.....	.....A..C.....A.....
Mouse	601	.....A.....T.....T.....T.....	.....C.....C.....C.....T..A.....T.....
DOG	721	GCCCGCTTGAGCAGGAGAGCGCCACGACTACAACCTTTGTCGCGCCTATGAATGCTTC	CAGCACAAGAACCCACAGTGGTCTTGTGAGATGTTGGAGCAGAACCTCTATGACTTT
Human	721	.....G.....T.....T.....C.....G.....C.....	.....C.....
Mouse	721	.....GC.....T.....T.....G..G.....G.....	.....A.....T.....
DOG	841	CTGAAGCAGAACAAAGTTTAGCCCTTGCCTCTCAATACATTGCGCCAGTCCACGAG	GTAGCCACAGCCCTGATGAACTGAAGAGCCTAGGTCTCATCCATGCCACCTCAAAACCA
Human	841	.....A.....T.....	.....C..A.....T.....C..T.....
Mouse	841	.....A.....T.....G..T.....	.....C..A.....G.....T.....
DOG	961	GAAACATCATGCTGGTAGATCCTTCCGACAGCCCTACAGAGTAAGGTCAATCGACTTC	GGTTCGGCCAGTCATGTGTCCAAGGCTGTGTGTTCCACCTACCTGCAGTCCAGATACTAC
Human	961	.....G.....A..TA.....A..A.....C.....T.....	.....A.....C..C.....C.....
Mouse	961	.....G.....A..A.....A..G..C.....G.....T.....T.....	.....A..T.....A.....C..C..T..G..T.....A.....
DOG	1081	AGGGCTCTGAGATCATCTTGGTTTACCATTTCGAGAGCAATTGATGTGTGCTCGTG	GGCTGTGTCATTGCTGAGCTGTTCTCGGCTGGCCGCTCTATCCAGAGCTCTCTGAGTAC
Human	1081	.....C.....T.....C.....	.....T.....A..AT.....T.....T..A.....G.....T.....
Mouse	1081	.....C.....G.....C.....T.....C.....T.....	.....T.....A.....AT..A..C.....T.....T.....
DOG	1201	GATCAGATTGATATATTTACAAACACAGGGTTTGCAGCTGAATATTTAATAGCGCA	GGGACAAAGACAAGTGTGTTTCAACCGTGACACAGACTCACCGTATCCCTTGTGGAGG
Human	1201	.....T.....C.....	.....G.....A.....T.....A.....
Mouse	1201	.....A.....T.....	.....T.....
DOG	1321	CTGAAGACACCATGACCATGAAGCAGAGACAGGGATTAAAGTGAAGAGCAAGAAAG	TACATATTCAACTGTTTAGATGACATGGCCAGGTGAACATGACAACAGATCTGGAGGG
Human	1321	.....T.....T.....	.....G.....
Mouse	1321	.....A.....G..A.....A..G.....	.....T.....G.....T.....T.....
DOG	1441	AGTGACATGTTGGTGGAGAGGAGAGTGGGAGTTTACCTGTTAAAGAGATG	CTGACCATAGATGCTGATAAGAGATCACTCCCATCGAAACCTGAACACCCCTTTGTC
Human	1441	.....C.....A..A.....T..C.....	.....T.....C.....A.....
Mouse	1441	.....A..A.....C..A.....	.....C.....G.....T.....T.....
DOG	1561	ACCATTGACACACCTCTCGATTTTCCACAGTACACAGCTCAATCATGTTCCAGAAC	ATGGAGATTTGCAAGCGTCGGTGAATATGTATGACACGGTGACCAAGGAGCAAAACCCCT
Human	1561	.....T..A.....C.....	.....C.....
Mouse	1561	.....G..T..C..C.....G..C..T.....T.....A.....	.....C.....C.....A.....A.....
DOG	1681	TTCATTACACATGTGGCCCCCAGCAGATCCACCAACCTGACCATGACCTTCAACAACAG	CTGAATCTGTCCACAACCGCTTCAGCGCGCTCCATGGCTGCAGTGGCTCAGCGGAGC
Human	1681	.....C..G..C.....G.....T.....	.....CC.....C.....A.....C.....
Mouse	1681	.....C..T..C.....T.....T.....	.....CC.....C.....A.....C.....G..C..C.....
DOG	1801	ATGCCCTGCAGACGGGAACAGCCAGATCTGTGCCCGGCTGACCCCTTCCAGCAAGCT	CTCATCGTGTGTCGCGCCGCTTCCAAGGTTGACGGCTCGCCCTCTAAGCACGCTGGC
Human	1801	.....A.....T.....	.....C.....
Mouse	1801	.....A..T.....A.....T.....	.....C..T.....CC.....T.....C.....
DOG	1921	TATTCGCTGAGAATGGAGAATGCGGTGCCATCGTCAAGCCCGGAGCAGCAGCT	CTTCAGATCCAACAGGTCTGCTTGGCCAGCAGGCTGGCCAAAGTGGAGCCAGCAAAATC
Human	1921	..C..G..C.....A.....A..T.....A.....T.....	.....T.....G.....
Mouse	1921	..C..A..C.G.....A.....T..C.....C..G..A.....T.....	.....C.....T.....G.....G.....A..G..
DOG	2041	CTGCTTCCCCGGCATGGCAGCAGTTGACCGAGTGGCCACCCACACATCCGTGCAGCAC	GCAACTGTGATTCCCGAGACCATGGCAGGACCCAGCAGTTGGCTGACTGGAGGAACAG
Human	2041	.....A.....AC.....T.....	.....C..C.....A..T.....
Mouse	2038	..A.....T..A.....C.....T..C.....T.....A.....T.....	.....G.....T.....T.....AC.....

**Fig. 1.** Alignment of the nucleotide sequence of canine HIPK2 cDNA with those of human, and mouse counterparts (GenBank accession numbers AF326592 and AF208292). Dots indicate regions of identities in nucleotides. Numbers on left indicate the nucleotide residue position. Gaps were introduced in sequences to maximize alignment (-). This canine HIPK2 cDNA sequence was deposited in the GeneBank nucleotide database under accession number AY800385.

DOG 3472 CAGTACCCTTACATATAA  
Human 3478 .....  
Mouse 3469 ..A.....

## Materials and Methods

A physically normal, middle-aged, mixed male dog was euthanized with 20 ml of thiopental sodium. Spinal cord was separated and stored until the mRNA extraction was conducted at  $-70^{\circ}\text{C}$  freezer.

The spinal tissue (30 mg) was disrupted in 1.5 ml tube with 350  $\mu$ l of lysis buffer (Macherey-Nagel, Germany) and was ground with automatic homogenizer. Total RNA was isolated from spinal tissue with RNA extraction kit (Macherey-Nagel, Germany). Full-length first strand cDNA was prepared from total RNA with First Strand cDNA Synthesis Kit (Fermentas, Lithuania). The cDNA was kept in  $-20^{\circ}\text{C}$  freezer.

region of human and murine nucleotide sequences (GenBank Accession No. AF326592 and AF208292). PCR reaction mixture was consisted with a pair of the primers (1.0  $\mu$ M each), Taq polymerase (0.75 units; TaKaRa, Japan), 10 $\times$  PCR buffer (10  $\mu$ l), dNTP mixture (8  $\mu$ l), template (1  $\mu$ g) and deionized water was added to a final volume of 25  $\mu$ l. Amplification was involved 35 cycles of denaturation (94°C, 1 min.), annealing (45~60°C, 1 min.) and polymerization (72°C, 2 min.) steps.

The PCR products were extracted by gel extraction kit-spin (NucleoGen, Korea) and were ligated into pCR2.1-TOPO vector (Invitrogen, USA). The vector was transformed into competent *E. coli* cells. Plasmid DNAs were isolated with plasmid purification kit (NucleoGen, Korea). The cloned plasmids were committed to TaKaRa-Korea Biomedical, in which ABI PRISM 377 sequencer is used to sequence analysis. The sequences were compared with

DOG	1	MASHVQVFSPTLOSSAFCSVKKLKVEPSSWMTGYGSHSKVYSQSKNVPPSPATTTV	STSLPIFNPSLPYEQTIFPGSTGHIIVTSASSTSVTGQVLGGPHNLMRRSTVSLDDTYQ
Human	1	.....N.....L.....V.....V.....	
Mouse	1	.....N.....L.....S.....	
DOG	121	KGLKRRKSEEIENTSSVQIIIEHPPMIQNNASGATVATATTSTATSKNSGNSGSDYQLV	QHEVLCSTMTNTYEVLEFLGRSTFGQVVKWKRGTEIVAIIKILKNHPSYARQGGIEVSI
Human	121	.....	
Mouse	121	.....	
DOG	241	ARLSTESADDYFVRAYECFQKHNTCLVFEMLEQNLYDFLKQNKFSPLPKYIRPVLLQ	VATALMKLKSGLIHADLKPENIMLVDPSPQPYRVKVIDFGSASHVSKAVCSTYLGSRYY
Human	241	.....	
Mouse	241	.....	
DOG	361	RAPEIILGLPFCEADIMNSLGCYIAELFLGWPLYPGASEYDQIRYISQTQGLPAEYLLSA	GTKTTRFFNRDTSPLYMLRLKTPDOHEAETGIIKSKEARKYIFNCLDDMAQVNMITDLEG
Human	361	.....	
Mouse	361	.....	
DOG	481	SDMLVEKADRRFIDLLKMLTIDADKRIPTIETLNHPFVTMTLLDFPHSTHVKSQFQN	MEICKRRVNMVDTVNSKTPFIHVAPSTSTNLMTFNNQNTVHNQPSAASMAVAQRS
Human	481	.....	.....T.....
Mouse	481	.....V.....A.....	.....T.....P.....
DOG	601	MPLQTGTAGICARPDPFQALIVCPGFGQLQASPSKHAGYSVRMENAVPIVTOAPGAQP	LQIQGLLAQQAIPSGTQQLLLPPAWQQLTGVAHTSVQGHATVIPETMAGTQQLADNRNT
Human	601	.....	.....G.A.....
Mouse	601	.....	.....A.....
DOG	720	HAHGSHYNPIMQPALLTGHVTLPAAGPLNVGVAHVMRQOPTSTSSRKSKQHSSARNV	STCEVSSQAISPPQSRKRVKENTPPRCALVHSSPACSSSVTCGWGASSTTRERQRT
Human	721	.....V.....	.....M.....T.....V.....
Mouse	720	.....V.....	.....M.....T.....V.....
DOG	840	IVIPDTPSPVSVITISSDTEEEQKHAPTSTVSKQKINVISCVTVHDSPYSDSSNITS	PYSVQHRAGHHG-NACDAKGSLENHCTGNPRTIIVPPLKQASEVLEQDSLVFVN-ASQ
Human	841	.....Q.....NNA.....F.T.....	.....T.H.....
Mouse	840	.....Q.T.....N.T.TL.T.G.....	.....G.AIS..H.....
DOG	959	HSS-YKSSSSNVTSTSGHSSGSSGAIAVYQQRPPGPHFQGGPLNLSQAQGHITAEAG	SHRRQAYITPTMAQAPYSFPHNSPSHGTVHPLAAAAAAHLPAGPHLYTYTAPALGS
Human	961	...S.....T.....TD.T.....	.....T.....
Mouse	960	...SF.....T.....MA.D.T.....	.....T.....T.....
DOG	1077	TGTVAHLVASQGSARHTVQHTAYPASIVHQVPSVSMGPRVLPSTIHPSSQYPAQFAHQTYI	SASPASTVYTGYPSPAKVNGYPI+
Human	1080	.....	
Mouse	1077	.....	

**Fig. 2.** The deduced amino acid sequences of canine HIPK2 were aligned with those of human and mouse. Dots indicate identities with amino acids of the canine HIPK2 sequence. Gaps were introduced in sequences to maximize alignment (-).

those of human and murine HIPK2 (GenBank Accession No. AF326592 and AF208292). The amino acid sequence of canine HIPK2 was deduced from nucleotide sequence.

## Results

About 30 pairs of primers were designed on the conserved region of human and murine HIPK2 in which 18 pairs of primers were used to find sequence of canine HIPK2. The other primers did not make PCR products or made different sequences products compared with human and murine HIPK2 sequences.

The clones which had overlapping regions were aligned to give a total sequence of 3489 bp as shown in Fig. 1. Canine HIPK2 cDNA sequence elucidated in this study was deposited in the GeneBank nucleotide database under accession number AY800385.

The identity between nucleotide sequence of canine HIPK2 and that of human and murine HIPK2 was 93% and 90%, respectively (Fig. 1). The identity between nucleotide sequence of human and mouse HIPK2 was 90%.

The canine HIPK2 cDNA contained an open reading frame encoding 1163 amino acid residues and the predicted amino acid sequence had 98% and 96% identity with those of human and mouse, respectively (Fig. 2). The nucleotide and amino acid sequences were highly conserved between

human, mouse and dog.

## Discussion

The nucleotide sequences of canine HIPK2 containing open reading frame region were found. The canine HIPK2 nucleotide sequence was similar to those of human and mouse. The deduced amino acid sequence of canine HIPK2 was also very similar to those of human and murine HIPK2.

The spinal cord was selected because HIPK2 mRNA was detectable in many tissues in human but a relatively high expression was observed in neural tissues, in which there are hippocampus, medulla oblongata, putamen, and so on [20]. Further study is needed to know where canine HIPK2 is expressed highly, using northern blot analysis, dot blot analysis, semi-quantitative RT-PCR [13,20].

HIPK2 contains multiple functional domains: an interaction domain for homeoproteins, a corepressor domain, a PEST sequence, a YH domain in the COOH-terminal and a protein kinase catalytic domain in the N-terminal side [10]. The enhancement of repressor activity of homeoproteins by HIPK2 is conferred by domains within the N-terminal half of the HIPK2. The SRS (nuclear speckle retention signal) that contains PEST sequence and YH domain has a positive and a negative effect on co-repressor activity respectively. It is expected that the functions of canine HIPK2 were similar

to those of human and murine HIPK2, because the deduced amino acid sequences of canine HIPK2 contained all these domains. For instance, HIPK2 acts as a transcriptional corepressor for homeoproteins and localizes to nuclear speckles. In the N-terminal of the catalytic domain there is a glycine-rich stretch of residues in the vicinity of a lysine residue, which has been shown to be involved in ATP binding. In the central part of the catalytic domain there is a conserved aspartic acid residue which is important for the catalytic activity of the enzyme.

HIPK2 has the function namely activation of transcription mediated by p53 specific promoter elements [19]. Overexpression of HIPK2 leads to an increase of the p53 protein level. The kinase defective mutant of HIPK2 leads to a decrease of p53 protein amounts. Overexpression of HIPK2 does not lead to a change of Mdm2 mRNA levels, but it leads to a downregulation of p53-induced Mdm2 protein.

In veterinary field, the attack rate of cancer is increasing due to the longevity of pets. So, the researches of cancer and p53 are highlighted and the study of HIPK2 may provide clinical benefits.

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