

# **Comparative genomics on HHIP family orthologs**

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Abstract. Hedgehog, FGF, VEGF, and Notch signaling pathways network together for vascular remodeling during embryogenesis and carcinogenesis. HHIP1 (HHIP) is an endogenous antagonist for SHH, IHH, and DHH. Here, comparative integromics analyses on HHIP family members were performed by using bioinformatics and human intelligence. HHIP1, HHIP2 (HHIPL1 or KIAA1822) and HHIP3 (HHIPL2 or KIAA1822L) constitute human HHIP gene family. Rat Hhip1, Hhip2, and Hhip3 genes were identified within AC107504.4, AC094820.6, and AC134264.2 genome sequences, respectively. HHIP-homologous (HIPH) domain with conserved 18 Cys residues was identified as the novel domain conserved among mammalian HHIP1, HHIP2, and HHIP3 orthologs. HHIP1 mRNA was expressed in coronary artery endothelial cells, prostate, and rhabdomyosarcoma. HHIP2 mRNA was expressed in trabecular bone cells. HHIP3 mRNA was expressed in testis, thyroid gland, osteoarthritic cartilarge, pancreatic cancer, and lung cancer. Promoters of HHIP family genes were not well conserved between human and rodents. Although GLI-, CSL-, and HES/HEY-binding sites were not identified, eleven bHLH-binding sites were identified within human HHIP1 promoter. Expression of HES/ HEY family members, including HES1, HES2, HES3, HES4, HES5, HES6, HES7, HEY1, HEY2 and HEYL, in coronary artery endothelial cells was not detected in silico. Up-regulation of HHIP1 due to down-regulation of Notch-CSL-HES/HEY signaling cascade repressing bHLH transcription factors results in down-regulation of the Hedgehog-VEGF-Notch signaling cascade. On the other hand, down-regulation of HHIP1 due to up-regulation of Notch signaling in vascular endothelial cells during angiogenesis results in up-regulation of the Hedgehog-VEGF-Notch signaling cascade. Because HHIP1 is the key molecule for vascular remodeling, HHIP1 is the

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pharmacogenomics target in the fields of oncology and vascular medicine.

## Introduction

Hedgehog signaling pathway is implicated in a variety of processes during embryogenesis, chronic persistent inflammation, and carcinogenesis (1-3). Hedgehog family of secreted proteins consists of Sonic Hedgehog (SHH), Indian Hedgehog (IHH) and Desert Hedgehog (DHH) (4-6). PTCH1, PTCH2, DISP1, DISP2 and DISP3 are multi-transmembrane proteins with two PTCH/DISP homologous domains (7,8). PTCH1 and PTCH2 are Hedgehog receptors, regulating the Hedgehog signal transducer Smoothened (SMO) (9-13). KIF27, KIF7, STK36, SUFU and DZIP1 are Hedgehog signaling components (10-17). GLI family transcription factors are implicated in the transcriptional activation of Hedgehog target genes, such as *PTCH1*, *CCND2*, *IGFBP6*, and *FOXM1* (1,18-20).

HHIP1 (HHIP) is secreted-type Hedgehog-interacting protein, functioning as an endogenous antagonist for SHH, IHH, and DHH (21). *HHIP1* expression is down-regulated in a variety of tumors, such as gastric, pancreatic, colorectal, esophageal and lung cancer (22,23). *HHIP1* down-regulation in pancreatic cancer is due to epigenetic CpG hypermethylation of *HHIP1* promoter.

In contrast to the down-regulation of *HHIP1* in various types of human tumors, *HHIP1* is abundantly expressed in human aortic endothelial cells (22). However, mechanism of *HHIP1* expression in human aortic endothelial cells remains unclear.

Recently, we identified two other members of human *HHIP* gene family. Here, comparative genomics analyses on *HHIP* family members as well as expression analyses of *HHIP* family members were performed. Because *HHIP1* was expressed in coronary artery endothelial cells, transcriptional mechanism of *HHIP1* in coronary artery endothelial cells was further investigated.

### Materials and methods

*Identification of novel genes*. Mouse cDNAs, ESTs, and rat genome sequences homologous to human HHIP1, HHIP2, and HHIP3 were searched for with the BLAST program (http://www.ncbi.nlm.nih.gov) as described previously (24,25). Exonintron boundaries were determined by examining the consensus sequence of exon-intron junctions ('gt ..... ag' rule of intronic

	Human					e	Rat	
Ģ	Gene	Alias	Chromosomal position	n Complete CDS	Complete	CDS	Complete CDS	
ŀ	HIP1	ннір	4q31.21	NM_022475.1	NM_020	259.3	This study	
ŀ	HIP2	KIAA 1822	14q32.2	NM_032425.3	AK1662	69.1	This study	
ŀ	ннгрз	KIAA 1822L	1q <b>41</b>	NM_024746.2	This st	udy	This study	
Exon		Bat Hhin1 a	ana	Rat Hhin2 gene			Rat Hhin3 gene	
Exon		Rat Hhip1 ge	ene CAAG gtaggc	Rat Hhip2 gene	G gtgagc		Rat <i>Hhip3</i> gene	gtgggt
01 02 03	tttcag	Rat Hhip1 go	CAAG gtaggc CCAG gtagaa ct GCCAG gtccat ct	Rat Hhip2 gene GGCCTTTGCCA acag GAATGCAGAGA ttag GATATAACA	G gtgagc G gtcgct A gtcgcc	ttccag	Rat Hhip3 gene GGCCAATGCCAG GAGTGCAGAGAG GGTCATGAACAA	gtgggt gtgaga gtaago
01 02 03 04	tttcag ttaaag gtttag	Rat Hhip1 go AGAAGCAR ATCTTTTT GTCTTCCR AAAGCAAT	CAAG gtaggc CCAG gtaaaa ct GCAG gtccat ct AAAG gttggc cc	Rat Hhip2 gene GGCCTTTGCCA acag GAATGCAGAGA ttag GATAATAAACA gcag GTCAGCCCCTC	G gtgagc G gtcgct A gtacgc G gtgacc	ttccag ttccag tttcag	Rat Hhip3 gene GGCCAATGCCAG GAGTGCAGAGAG GGTCATGAACAA AAGTTCGAACAA	gtgggt gtgaga gtaagc gtaagt
01 02 03 04 05	tttcag ttaaag gtttag ctgcag	Rat Hhip1 go	CAAG gtaggc CCAG gtaaaa ct GCAG gtccat ct 'AAAG gtcgc cc 'CCAG gtatca cc	Rat Hhip2 gene GGCCTTTGCCA acag GAATGCAGAGA ttag GATAATAAACA gcag GCAACCCCTC tcag ATGACGGAACG	G gtgagc G gtcgct A gtacgc G gtgacc G gtgacgt	ttccag ttccag tttcag tgacag	Rat Hhip3 gene GGCCAATGCCAG GAGTGCAGAGAG GGTCATGAACAA AAGTTCCCTTGG ACCACAGAGTGG	gtgggt gtgaga gtaagc gtaagt gtaaga
01 02 03 04 05 06 07	tttcag ttaaag gtttag ctgcag atgcag	Rat Hhip1 go	Ene CCAAG gtaggc CCAG gtaaaa ct GCAG gtccat ct AAAG gttggc cc CCAG gtatca cc TAAG gtaaca tt KCAG gtaaca tt	Rat Hhip2 gene GGCCTTTGCCA acag GAATGCAGAGA ttag GATAATAGAGA gcag GTCAGCCGCCG ccag GCGACTAGGCT acag GCGACCCTCGA	G gtgagc G gtcgct A gtacgc G gtgacc G gtagt G gtgagc G gtagt	ttccag ttccag tttcag tgacag atctag ccttag	Rat Hhip3 gene GGCCAATGCCAG GAGTGCAGAGAG GGTCATGAACAA AAGTTCCCTTGG ACGACAAAGCCG GGGBCCAAGCCG	gtgggt gtgaga gtaago gtaaga gtaaga gtaaga
01 02 03 04 05 06 07 08	tttcag ttaaag gtttag ctgcag atgcag tctcag atgcag	Rat Hhip1 ge	CAAG gtaggc CCAG gtagaa ct GCAG gtccat ct AAAG gttggc cc CCAG gtatca cc TAAG gtaaca tt GCAG gtgagg tc TACG gtgagg tc	Rat Hhip2 gene GGCCTTTGCCAA acag GAATGCAGAGA ttag GATAATAAGAGA ttag GTCAGCCCCTC tcag ATGACGGAGCGC ccag GCGACTAGGAC gcag ACGGCAGGAC	G gtgagc G gtogot A gtacgc G gtgacc G gtagt G gtgagt G gtgagt A gtagt	ttccag ttccag tttcag tgacag atctag ccttag ttacag	Rat Hhip3 gene GGCCAATGCCAG GAGTGCAGAGAG GGTCATGAACAA AAGTCCTTGG ACGACACTCAG GGGAGCCTCAAG GGGAGCCTCAAG	gtgggt gtgaga gtaago gtaagt gtaaga gtaact gtgaga
01 02 03 04 05 06 07 08 09	tttcag ttaaag gtttag ctgcag atgcag tctcag atgcag ttccag	Rat Hhip1 ge	CAAG gtaggc CCAG gtaaaa ct GCAG gtccat ct AAAG gttggc cc CCAG gtatca cc TAAG gtagg tc TAAG gtgagg tc TACG gtgagt tg AITGG gtgtgt gt	Rat Hhip2 gene GGCCTTTGCCA acag GAATCCAGAGA ttag GATAATAAACA gcag GTCACCCCCTC tcag ATGACGCACCGC acag GCGACCAGGCT acag GGGACCAGGAA gcag ACGGCAGGAA	G gtgagc G gtcgct A gtacgc G gtgacc G gtagt G gtgagc G gtgagt A gtagt C	ttccag ttccag tttcag tgacag atctag ccttag ttacag ttccag	Rat Hhip3 gene GGCCAATGCCAG GAGTGCAGAGAG GGTCATGAACAA AAGTCCTTGG ACGACAGAGTGG TCGACTAAGCCG GGGAGCCTCAAG GCGAGCGCGCAG CCACAGGCACA	gtgggt gtgaga gtaaga gtaaga gtaact gtgaga gtgaga
01 02 03 04 05 06 07 08 09 10	tttcag ttaaag gtttag ctgcag atgcag tctcag atgcag ttccag tcatag	Rat Hhip1 gd AGAAGCAR ATCTTTTT GTCTTCCA ANAGCAAT GAAGAAGT TGATTTAT ANAGCGAT ANAGCGCA GAATTTAR	CAAG gtaggc CCAG gtaaaa ct GCAG gtccat ct AAAG gttggc cc CCAG gtatca cc TAAG gtatca cc TAAG gtgagg tc TACG gtgaggt tg AATGG gtggtgt gt ITTAG gtatcg	Rat Hhip2 gene GGCCTTTGCCA acag GAATGCAGAGA ttag GATATAAACA gcag GTCACCCCCTC tcag GGAGCCGCGC ccag GCGACTAGCCT acag GGGAGCAGCAA gcag ACGGCCAGCAA gtag AGTTCAATTTT	G gtgage G gtcgct A gtacgc G gtgagc G gtgagc G gtgagc G gtgagt A gtaagt C	ttccag ttccag tttcag tgacag atctag ccttag ttacag ttccag	Rat Hhip3 gene GGCCAATGCCAG GAGTGCAGAGAG GGTCATGAACAA AAGTTCCCTTGG ACGACAGAGTGG GGGAGCAGCCG GGGAGCGCGCAG CCACAGATAAAT	gtgggt gtgaga gtaago gtaagt gtaaga gtaact gtgaga gtgagt
01 02 03 04 05 06 07 08 09 10 11	tttcag ttaaag gtttag ctgcag tctcag atgcag tcccag tcccag tcatag caacag	Rat Hhip1 gd AGAAGCAR ATCTTTTT GTCTTCCA AAAGCAAT GAAAAAGT TGATTTAG ATGTGCAT AAAGCGCA GAATTTAR GAAGGGAR	CAAG gtaggc CCAG gtaaaa ct GCAG gtccat ct 'AAAG gtaggc cc CCAG gtatca cc 'TAAG gtaaca tt GCAG gtgagg tc 'TACG gtgagg tg IATGG gtgtgt gt ITTAG gtatcg AAAG gtaaga	Rat Hhip2 gene GGCCTTTGCCA acag GAATGCAGAGA ttag GATAATAAACA gcag GTCAGCGAGCG ccag GCGACTAGGCT aaag GGGACCCTCCA gcag ACGGCCAGGAA gtag AGTTCAATTTT	G gtgagc G gtcgct A gtacgc G gtgacc G gtgagt G gtgagc G gtgagt A gtaagt C	ttccag ttccag ttccag tgacag atctag ccttag ttacag ttccag	Rat Hhip3 gene GGCCAATGCCAG GAGTGCAGAGAG GGTCATGAACAA AAGTTCGAGTGG TCGACAGAGTGG TCGACTAGCCG GGGAGCCCAAG GCGAGCGCGCAG CCACAGATAAAT	gtgggt gtgagg gtaagg gtaagt gtaagg gtaact gtgagg gtgagt

Figure 1. (A) Mammalian HHIP gene family consisting of HHIP1, HHIP2 and HHIP3 orthologs. (B) Exon-intron structure of rat Hhip1, Hhip2, and Hhip3 genes.

sequence) and the codon usage within the coding region as described previously (26,27).

*Comparative proteomics analyses*. Translation into amino-acid sequence and amino-acid alignment were performed with the Genetyx program as described previously (28,29). Signal peptide and transmembrane domain were searched for with the Kyte and Doolittle hydrophobicity analysis and PSORT II program. Domain architecture was at first searched for with the RPS-BLAST program (http://www.ncbi.nlm.nih.gov) as described previously (30,31). Novel domains were then searched for based on the conservation among related proteins as described previously (32,33).

*Comparative genomics analyses.* Genome sequences around human *HHIP1*, *HHIP2* and *HHIP3* genes were used as query sequences for the BLAST program to identify evolutionarily conserved regions. Transcription factor-binding sites within the promoter region were searched for with the Match program (http://www.gene-regulation.com) as well as by manual inspection.

## Results

*Human HHIP family genes.* BLAST program using HHIP1 amino-acid sequence NP\_071920.1 as a query sequence revealed that NM\_032425.3 and NM\_024746.2 RefSeqs were derived from human *HHIP1*-related genes. Human genes corresponding to NM\_032425.3 and NM\_024746.2 were designated *HHIP2 (HHIPL1 or KIAA1822)* and *HHIP3 (HHIPL2 or KIAA1822L)*, respectively (Fig. 1A).

Preliminary alignment of HHIP family members revealed that 529-aa NP\_079022.1 was N-terminally truncated HHIP3 protein. Although nucleotide position 644-2233 of NM\_024746.2 RefSeq was translated for NP\_079022.1, we found that nucleotide position 59-2233 was the real coding region. Instead of 529-aa N-terminally truncated HHIP3 partial amino-

acid sequence, 724-aa full-length HHIP3 amino-acid sequence translated from the real coding region of NM\_024746.2 RefSeq was used for the following study.

*Mouse Hhip family genes*. Mouse cDNAs homologous to human HHIP1, HHIP2, and HHIP3 were searched for with BLAST programs. Mouse NM\_020259.3 RefSeq, AK166269 cDNA, and NM\_030175.1 RefSeq were derived from mouse *Hhip1*, *Hhip2*, and *Hhip3* genes, respectively. NM\_020259.3 RefSeq and AK166269 cDNA were representative full-length clones; however, NM\_030175.1 RefSeq was a 5'-truncated partial clone (Fig. 1A).

BE305786 EST, corresponding to the 5'-UTR and Nterminal part of coding region of Hhip3, overlapped with NM\_030175.1 5'-truncated partial RefSeq. Mouse Hhip3 complete CDS was determined by assembling BE305786 EST and 5'-truncated NM\_030175.1 RefSeq (Fig. 1A).

*Rat Hhip family genes*. Rat *Hhip1*, *Hhip2* and *Hhip3* genes were identified within AC107504.4, AC094820.6 and AC134264.2 genome sequences, respectively. Exon-intron boundaries of rat *Hhip1*, *Hhip2* and *Hhip3* genes were determined by examining the consensus sequence of exon-intron junctions and the codon usage. Rat *Hhip1*, *Hhip2* and *Hhip3* genes were found to consist of 13, 9, and 9 exons, respectively (Fig. 1B). Complete CDSs of rat Hhip1, Hhip2 and Hhip3 and Hhip3 were determined by assembling exonic regions. Rat *Hhip1*, *Hhip2* and *Hhip3* genes were found to encode 700-, 791- and 712-amino-acid proteins, respectively (Fig. 2).

*Comparative proteomics on HHIP family members*. Membrane topology analyses were performed at first. HHIP1 and HHIP2 orthologs were secreted proteins with N-terminal signal peptide, while HHIP3 orthologs were type II transmembrane proteins with short N-terminal cytoplasmic region.

Human, rat, and mouse HHIP family members were then aligned for comparative proteomics analyses. Although N- and

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3.	PUBLICATION	SikmisfklillavalgffegdakfgernegsgarrrclngnppkrikrrdrrmmsQ-lelisggenlcggfypriscclrsdspgig-rlenkifsvinntecgklieeikcalcsphsosifhsperevie	132
Ÿ	100000000000000000000000000000000000000	LKMLSFKLLLLAVALGFFEGDAKFGERSEGSGARRRRCLNGSPFKRLKRRDRRMSQ-LELLSGGEILCGGFYPRVSCCLQSDSPGLG-RLENKI~-FSATNNTECGRLLEEIKCAPCSPHSOSLFFSPERDVLD	132
	мш натрт	MLKMLSFKLLLLAVALGFFEGDAKFGERNEGSGARRRRCLNGNPFKRLKRRDRRVMSQ-LELLSGGEILCGGFYPRVSCCLQSDSPGLG-RLENKI-FSATNNSECSRLLEEIOCAPCSPESOSLFYTPERDVLD	132
	Rs HHIP2	MARARAGALLALWVLGAAA	104
	Rn Hhip2	MARORTVAGIGPGALLALRALLVAA	110
	Mm Hhip2	MAGRRAVARIGPGALLVLRALLAAA	110
	Es HHIP3	MLRTST-PNLCGGLHCRAPWLSSGILCLCLIFLLGOVGLLOGHPOCLDYGPPFOPPLHLEFCSD-YESFGCCDOHEDRIAARYWDIMEYFDLERHELCGDYYEDILCOECSPYAAHLYDAENTOTDL	120
	Rn Hhip3	MLGKERSPHTVSGRPAOWLSPGIFCLGLTFLLGWVGLLOG	125
	Mm Hhip3		125
		+ + + +	
	Hs HHIP1		270
	Rn Hhipl	GDLALPLLCKDYCKEFFYTCRGEIPGLLOTTAD-FFCFYYAREDAGLCFPDFPRKOVRGPASNYLDOMENYEKVETSREKENG-FCVGFVMGGLODAGUSGLOGGGGGGGLDIFLEFEGYVETTTPGGEVETT	270
	Mm Hhip1	GDLALPLLCKDYCKEFFYTCRGHIPGLLOTTAD-EFCFYYARKDAGLCFPDFPRKOVRGPASNYLGOMEDYEKVGGISREHKENC-LCVOFYNGGIPGDYBAVRGCDGHDIFTIFKFGYVFIFTDFOFT FFFFYYDTDHK	270
	Es HHIP2	RTVPGLCODYCLDMWHKCRG-LFRHLSTDOELWALEGNLARF-CRYL-SMDDTDYCFPYLLUNKNINGNUVADARG-CLOLCLEEVANGLANDAWDAPDGTEFFUAFOVGLWAYLAPDGFTGTTTTT	2 3 3 3
	Rn Hhip2	RTVPGLCEDYCLDMWOTCRG-LFRHLSPDRELWALEANRAKI-CRVI-SIDDTDYCEPSILUNENINGNIGRUVADAGG_CLOICI.EVANGLWVADAGGGGGGFFFVAFOVGLWWVIDDD961 FFDFINICO	247
	Mm Hhip2	RTVPGLCEDYCLDMWOTCRG-LFRLLSPDRELWALESNRAKI-CRVI-SLDDTDYCFPSLLUNENINSNLGRVVADAKG-CLOLCLERVANGLENDVAMVABGDGWBFFVAFOVALWWYLDDDSDLFTDFINVSO	241
	HS HHIP3	RN-LPGLCSDYCSAFHSNCRS-AISIL-TNDRGLOESHGRDGTRECHL, DIDEDYCPDNU, BNDYLDNHGAWAODOGAC, CLOICLSRWANG, DWGWWADDOGAW FYWAFWAUW DAGAWAFTWA FWAWWY DAGAWAFTWA	244
	Rn Hhip3	RNLPGLCSDYCSAFHENCES-ATSLLTNDRGLOESEGEDGARFCHLL-NLDEDYCFPNVLRNDLMNLGVVAFDEGG_CLOLCLSEVANCENDVGMVEAGDCHEFVXEQUVVAFTLDGSRLEGFTELDERN	200
	Mm Hhip3		255
		* ** *** * * * * * * * * * * * * * * *	205
	HS HHIP1		407
	Rn Hhipl	LVOSGIKGGDERGLISLAFEDNYKKNGKLVVSYTTNOERNA IGPENDILEVVEYTVABENDEVVIDERA PULLEVAFLADEN GADI I FODDAFI VI I I GOMITE DOMERA DO GENTALDAVIDE VIDERA VIDER	407
	Mm Hhip1	LVOSGIKGGDERGLISLAFEPNYKKNGKLVVSYTTNOERMAIGPEDHILEVVEYTVSEKNPEONDURTARVELEVAELSEGULISLAFEPNYKKNGKLVVSYTTNOERMAIGPEDHILEVVEYTVSEKNPEONDURTARVELSEGULISLAFEFNGAGULISLAFEPNYKKNGKLVVSYTTNOERMAIGPEDHILEVVEYTVSEKNPEONDURTARVELSEGULISLAFEFNGAGULISLAFEPNYKKNGKLVVSYTTNOERMAIGPEDHILEVVEYTVSEKNPEONDURTARVELSEGULISLAFEFNGAGULISLAFEFN	407
	HS HHIP2	VVI.TSPWEGDERGELGIAFEPAFORNERI.VVVYSVGIDSEWI_DISEPPUGEDDENAUNDEGEDIII.EVVEDANUUGALIEDDAUVI.VVTSDWEGDEGEGUSAUNDEGEVEDUSEL	271
	Rn Hhip2	AVLTEDWEGDERGFLGLAFFDEPDEPSKLVVVSVGVGFEWI_ETSEPDENTVDUGSERTILETEDANUNGGGLEGDDGTTILGUGGGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGAGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGGAGDEGEFGGTAGGAGAGDEGEFGGTAGGAGGAGDEGEFGGTAGGAGGAGDEGEFGGTAGGAGGAGDEGEFGGTAGGAGGAGDEGEFGGTAGGAGGAGDEGEFGGTAGGAGGAGGGTAGGGGTAGGGGTAGGGGTAGGGGTAGGGGGG	371
	Mm Hhip2	AVLTSPWEGDERGFLGLAFEPEFPEPSKLVVVSKGVGFBEWI_ETSEFPUSEDENT/DEGSETILIFIEDAMENGGGLIFGDBGFTVFFGGAGAADDEGSETGANONSALLGVVLKTVVSKGVGF	277
	HS HHTP3	IVI.TTWIGDERGFIGI.BFWDERFHNBEFVIVYSGIDEEEWEI_BIGPWEVGDADDNEADIESEVITIEEEDSUURGGIDFGDEGUEGDEGUEGDGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDDWEGAUGUEGDUGGADDDEGEGUGDWEGAUGUEGDUGGADDDEGEGUGGADDDGGGUGGGUGGADDDEGEGUGGADDDGGADDDEGEGUGGADDDGGGUGGADDDEGGUGGADDDEGGUGGADDDGGGUGGADDDGGGUGGADDDEGGUGGADDDGGGUGGADDDEGGUGGADDDGGGUGGADDDGGGUGGADDDGGGUGGADDDGGGUGGADDDGGGUGGGU	311
	Rn Hhin3	WU.TTPWIGDERGFIGIAFFEFFENDEFFIVES/LARDE	390
	Mm Hhip3	WULTTPWIGDERGFLGLAFFPEFPENDEFVIVYSGLGERE VET- PISTEVELSINGUAL TEDEPANDAGAGU FOLDUGULIFI GUDGGAGDEFOK PEREKANAGUL GULU TUNGAGAGU T	393
	···· ·····		293
		+ +	
	Hs HHIP1		622
	Rn Hhip1	IPRSNPHFNSTNOP-PEVFABGLEDPGRCAVDRH-PTDININITICSDENGENESS-APTIOTIK-GPD-VE-GPD-VE-FFFBLLEFK-FFBLMAPLY-GFWV9GCOFFLYGAVEDDA GWFLFLI-DG	522
	Mm Hhip1	IPRSNPHFNSTNOP-PEVFABGLEDPGRCAVDRE-PTDININITILCSDENGENES-APTIOTIC-CORP. VICE PERSIDE APTIC ACTIVACCORPT VCCVVPADDA APTIC	522
	Hs HHIP2	IPPDNP-FVGDPAAOPEVVALGVENMWRCSFDRGDPSSGTGRGRIFCGDV-GONFFEUDVVE-RGGNVGWRAPEGFEVDPSICANTEINDILDIE VERDVUGGVVVGGVVVGGVVVGGVVPAGVVVGGVVPAGVVVGGVVPAGVVVGGVVPAGVVVGGVVPAG	544
	Rn Hhip2	IPLDNP-FVDDPEARPEVYALGVRNMWRCSFDRGDPVSGTHRGRIFCGDV-GONRYEEVDIVE-RGRNVGWRAREGFEVYDRICANASI.DUU.DIR HUNDVGGVVRGVVRGVVRGVVRGVVRGVVRGVVRGVVRGVVRG	511
	Mm Hhip2	IPPDNP-FVDDPGARPEVYALGVRNMWRCSFDRGDPMSGTGRGRLFCGDV-GONRYEEVDLVERGRNYGWRAREGFEVYDRI.CANTALDUV.PTPAPHELGESVTGGVVVGGVVPGGVVPGGVVPGGVVPGGVVPGGVVPGG	513
	Hs HHIP3		632
	Rn Hhip3	VPLDNP-FVSEPGAHPAVYAYGVRNMWRCAVDRGDPVTROGRGRIFCGDV-GONFFEEVDLIVKGGNYGWRAEEGFEYDKNCHNASLDDILPIYAYGHQVASSYTGGYVRGCEAPNINGLYTFODFMGAP-IMALO	531
	Mm Hhip3		531
	-		551
		+ +	
	Hs HHIP1	QSPVTKQWQEKPLCLGTSGSCRGYFSGHILGFGEDELGEVYILSSSK6MTQTHNGKLYKIVDPKRPLMPEECRATVOPAOTLTSECSRLCRNGYCTPTGKCCCSP	627
	Rn Hhipl	QSPVTKQWQEKPLCLGASGSCQGYFSGHILGFGEDELGEVYILSSSKSMTOTHNGKLYKIIDPKRPLMPEECRVTVOPAOTLTSDISBLCRWGYYTPTGKCCCSP	627
	Mm Hhip1	QSPVTRQWQEKPLCLGASSSCRGYFSGBILGFGEDELGEVYILSSSKSMTQTHNGKLYKIVDPKRPLMPEECRVTVOPAOPLTSDCSRLCRNGYYTPTGKCCCSP	627
	Hs HHIP2	ENPGTQQWQYSEICMGHGQTCEFPGLINNYYP-YIISFGEDEAGELYFMSTGEPSATAPRGVVYKIIDASSCKARSAMPGYVPAPSVCSSLTSO	600
	Rn Hhip2	ENPESGQWKYSEVCMGRGQTCAFPGLINNYYP-YIISFAEDEAGELYFMSTGVPSATAARGVIYKVTDPSRRAPPGKCKIRPAQVKVKSELIFFVPKEKFIRTSESTPRPTARAPTKAPRRRPTAAPPAPTI.RPTKPARP	653
	Mm Hhip2	ENPETGQWKYSEVCMGRGQTCAFPGLINNYYP-YIISFAEDEAGELYFMSTGVPSATAAHGVIYKVIDPSRRAPPGKCKIRPAQVKVRSHLIFFVPKEKFIRTPESTPRPTARAPTKAPRRRPTAAPPAPTPRPTKPARP	653
	Hs HHIP3	EDRKNKKWKKQDLCLGSTTSCAFPGLISTESK-FIISFAEDEAGELYFLATSYPSAYAPRGSIYKFVDPSRRAPPGKCKYKPVPVRTKSKRIPFRPLAKTVLDLLKEOSEKAARKSSSATLASGPAOGLSE	662
	Rn Hhip3	EDRKTQKWSKRDICLGNSS-CAFPGLISAYSK-FIISFAEDEAGELYFLATSYPSAYAPHGSIYKFVDPSRRAPPGKCKLKPVPVRAKSKKIRFRPRAATVLDSMKEESOKAAGKPSTSTLASS5DRAAPO	660
	Mm Hhip3	EDRKTQKWTKRDICLGNST-CAFPGLISAYSR-FIISFAEDEAGELYFLATSYPSAYAPHGSIYKFVDPSRRAPPGKCKYKPVPVKTKSKKVRFRPLAATVLDLLKEESOKAARKASNATFTSSSDRVASO	660
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	Hs HHIP1	GWEGDFCRTAKCEPACRHGGVCVRPNKCLCKKGYLGPQCEQVDRNIRRVTRAGILDQIIDMTSYLLDLTSYIV	700
	Rn Hhipl	GWEGDFCRIAKCEPPCREGGVCVRPNKCLCKKGYLGPQCERADRSMRRVTRAGILDQIIDVTSYLLDLTSYIV	700
	Mm Hhip1	GWEGDFCRIAKCEPACREGGVCVRPNKCLCKKGYLGPQCEQVDRNVRRVTRAGILDQIIDMTSYLLDLTSYIV	700
	Hs HHIP2	PFILQWWK	608
	Rn Hhip2	TRRPGARKGGGRRRGRPGTAVPEPQNGSVRLVRPSGLSPGRGRVEVFIGGR%GTVCDDG%DIKAAAVVCRQLGFAHAVRAAKRAEFGEGRALRILLDDVRCAGSERNLLECAHAGVGTHNCKHEEDAGVECSHEDPDL	791
	Mm Hhip2	TRRPGARKGGGRRRGRPSTAVPEPENGSVRLVRPAGLSPGRGRR-GSVERRTLGEGV	709
	Hs HHIP3	KGSSKKLASPTSSKNTLRGPGTKKKARVGPHVRQGKRRKSLKSESGRMRPSAEQKRAGRSLP	724
	Rn Hhip3	RGSPKKPASPPSSKKTFQRPSTKKKSRMWSPGPQGKRKQSQATRKSPGRSEP	712
	Mm Hhip3	KGSLKKPASSRSSKKTFRRPGTKKKSRVWSPRPQGKRKPNLDSEGVGMRQAAGRSEP	717

Figure 2. Alignment of vertebrate HHIP1, HHIP2 and HHIP3 orthologs. Hs, human. Rn, rat. Mm, mouse. HHIP homologous (HIPH) domain is boxed. Conserved Cys residues within the HIPH domain are shown by a cross.

C-terminal region of HHIP family members were divergent, core region corresponding to codon 68-595 of human HHIP1 was well conserved among mammalian HHIP family members (Fig. 2). The novel conserved region with 18 conserved Cys residues was designated the HHIP-homologous (HIPH) domain. These facts indicate that HHIP family members should be characterized as HIPH domain proteins.

Differential expression of HHIP1, HHIP2 and HHIP3 mRNAs. ESTs corresponding to HHIP1, HHIP2, and HHIP3 mRNAs were searched for by using the BLAST program. The sources of ESTs were then listed up for the *in silico* expression analyses. HHIP1 mRNA was expressed in coronary artery endothelial cells, prostate, and rhabdomyosarcoma. HHIP2 mRNA was expressed in trabecular bone cells. HHIP3 mRNA was expressed in testis, thyroid gland, osteoarthritic cartilarge, pancreatic cancer, and lung cancer.

*Comparative genomics on HHIP1, HHIP2 and HHIP3 orthologs.* BLAST program as well as in house alignment of 5'-flanking regions revealed that *HHIP1, HHIP2* and *HHIP3* promoters were not well conserved between human and rodents.

Transcriptional regulation of human HHIP1 in coronary artery endothelial cells. We next analyzed the human HHIP1 promoter to elucidate the mechanism for HHIP1 expression in coronary artery endothelial cells. GLI family transcription factors, TCF/LEF family transcription factors, and CSL transcription factor are implicated in the transcriptional regulation of Hedgehog, WNT, and Notch target genes, respectively (1,34-38). Because GLI-, TCF/LEF-, and CSL-binding sites were not identified within the human *HHIP1* promoter (Fig. 3A), *HHIP1* was not the direct transcriptional target gene of Hedgehog, WNT, and Notch signaling pathways.

Among *HES/HEY* family genes encoding transcriptional repressors with bHLH and orange domains, including *HES1*, *HES2*, *HES3*, *HES4*, *HES5*, *HES6*, *HES7*, *HEY1*, *HEY2* and *HEYL* (39,40), at least *HES1*, *HES5*, *HES7*, *HEY1*, *HEY2* and *HEYL* are best characterized Notch target genes. Expression of *HES/HEY* family members was not detected in coronary artery endothelial cells by using *in silico* expression analyses.

Eleven bHLH-binding E-boxes were identified within human *HHIP1* promoter, while HES/HEY-binding N-box was not identified within human *HHIP1* promoter (Fig. 3A). Because HES/HEY transcription factors repress bHLH factors, down-regulation of HES/HEY expression leads to up-regulation of *HHIP1* mRNA depending on bHLH transcription factors (Fig. 3B).

## Discussion

Mammalian HHIP family members were comprehensively identified and characterized in this study (Fig. 1). Complete



Figure 3. Regulation of *HHIP1* transcription in vascular endothelial cells. (A) Human *HHIP1* promoter. Exon 1 of human *HHIP1* gene is boxed. Eleven bHLHbinding sites within human *HHIP1* promoter are shown by double over-lines. (B) HHIP1 and Hedgehog-VEGF-Notch signaling cascade. Up-regulation of HHIP1 due to down-regulation of Notch-CSL-HES/HEY signaling cascade repressing bHLH transcription factors results in down-regulation of the Hedgehog-VEGF-Notch signaling cascade. On the other hand, down-regulation of HHIP1 due to up-regulation of Notch signaling in vascular endothelial cells during angiogenesis results in up-regulation of the Hedgehog-VEGF-Notch signaling cascade.

CDS of mouse Hhip3 was determined by assembling BE305786 EST and 5'-truncated NM\_030175.1 RefSeq. Complete CDS of rat Hhip1, Hhip2, and Hhip3 were determined by assembling exonic regions within AC107504.4, AC094820.6, and AC134264.2 rat genome sequences, respectively (Fig. 1B). Comparative proteomics analyses revealed that HIPH domain with 18 conserved Cys residues was conserved among mammalian HHIP1, HHIP2, and HHIP3 orthologs (Fig. 2).

*HHIP1* mRNA was expressed in coronary artery endothelial cells, prostate, and rhabdomyosarcoma. *HHIP2* mRNA was expressed in trabecular bone. *HHIP3* mRNA was expressed in testis, thyroid gland, osteoarthritic cartilarge, pancreatic cancer, and lung cancer. Because preferential expression of *HHIP1* mRNA in coronary artery endothelial cells was the most interesting fact obtained by expression analyses, transcriptional mechanism of *HHIP1* mRNA in coronary artery endothelial cells was further investigated.

Notch signaling pathway is implicated in artery morphogenesis during embryogenesis as well as angiogenesis during carcinogenesis (34,35), and *HES1*, *HES5*, *HES7*, *HEY1*, *HEY2*, and *HEYL* are Notch target genes in vascular endothelial cells. Although we can not completely deny the false negativity based on *in silico* expression analyses, expression of *HES*/ *HEY* family members in coronary artery endothelial cells was not detected in this study. These facts indicate that the Notch-CSL-HES/HEY signaling cascade was down-regulated in human coronary artery endothelial cells.

HES/HEY family members are repressors for bHLH transcription factors. Eleven bHLH-binding sites were identified within human *HHIP1* promoter (Fig. 3A), five bHLH-binding sites within rat *Hhip1* promoter, and two bHLH-binding sites within mouse *Hhip1* promoter. Down-regulation of the Notch-CSL-HES/HEY signaling cascade in coronary artery endothelial cells leads to *HHIP1* up-regulation depending on bHLH transcription factors (Fig. 3B). VEGF-induced expression of DLL4 in vascular endothelial cells leads to the activation of Notch signaling (41). Notch signaling activation leads to up-regulation of HES/ HEY family members, and the following down-regulation of HHIP1 (Fig. 3B). HHIP1 down-regulation then leads to SHH activation (21), which results in the activation of VEGF signaling (42). Therefore, VEGF-induced downregulation of HHIP1 during angiogenesis leads to the positive feedback to the Hedgehog-VEGF-Notch signaling cascade (Fig. 3B).

Expression level of HHIP1 affects vascular remodeling through the regulation of Hedgehog-VEGF-Notch signaling cascade. HHIP1 itself could be utilized for anticancer agent as the Hedgehog inhibitor. On the other hand, monoclonal antibody, RNAi compound, and small-molecule compound down-regulating HHIP1 function could enhance the angiogenic effects of VEGF and FGFs for coronary artery disease. HHIP1 is the pharmacogenomics target in the fields of oncology and vascular medicine.

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