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 pharmacogenomics target in the fields of oncology and vascular medicine.

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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 hyper-methylation 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). Exon-intron boundaries were determined by examining the consensus sequence of exon-intron junctions ('gt ..... ag' rule of intronic

A

	Human			Mouse	Rat
Gene	Alias	Chromosomal position	Complete CDS	Complete CDS	Complete CDS
HHIP1	*HHIP*	4q31.21	NM_022475.1	NM_020259.3	This study
HHIP2	*KIAA1822*	14q32.2	NM_032425.3	AK166269.1	This study
HHIP3	*KIAA1822L*	1q41	NM_024746.2	This study	This study

B

Exon	Rat *Hhip1* gene	Rat *Hhip2* gene	Rat *Hhip3* gene
01	AGAAGC------AACAAG gtaggc	GGCCTT------TGCCAG gtgagc	GGCCAA------TGCCAG gtgggt
02	tttcag ATCTTT------TTCCAG gtaaaa	ctacag GAATGC------AGAGAG gtcgct	ttccag GAGTGC------AGAGAG gtgaga
03	ttaaag GTCTTC------CAGCAG gtccat	ctttag GATAAT------AAACAA gtacgc	ttccag GGTCAT------GAACAA gtaagc
04	gtttag AAAGCA------ATAAAG gttggc	ccgcag GTCAGC------CCCTCG gtgacc	tttcag AAGTTC------CCTTGG gtaagt
05	ctgcag GGAGGA------ATCCAG gtatca	cctcag ATGACG------GAGCGG gtaagt	tgacag ACGACA------GAGTGG gtaaga
06	atgcag GAAAAA------GTTAAG gtaaca	ttccag GCGACT------AGGCTG gtgagc	atctag TCGACT------AAGCCG gtaact
07	tctcag TGATTT------AGGCAG gtgagg	tcaaag GGGAGC------CTCCAG gtgagt	ccttag GGGAGC------CTCAAG gtgaga
08	atgcag ATGTGC------ATTACG gtgagt	tggcag ACGGGC------AGGAAA gtaagt	ttacag GCGAGC------GCGCAG gtgagt
09	ttccag AAAGCG------CAATGG gtgtgt	gtgtag AGTTCA------ATTTTC	ttccag CCACAG------ATAAAT
10	tcatag GAATTT------AATTAG gtatcg		
11	caacag GAGAGG------AAAAAG gtaaga		
12	atgcag ACCTTT------GAATTG gttagt		
13	ttgcag CCAAAT------TGAGAG		

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 N-terminal 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 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

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 transcsription 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

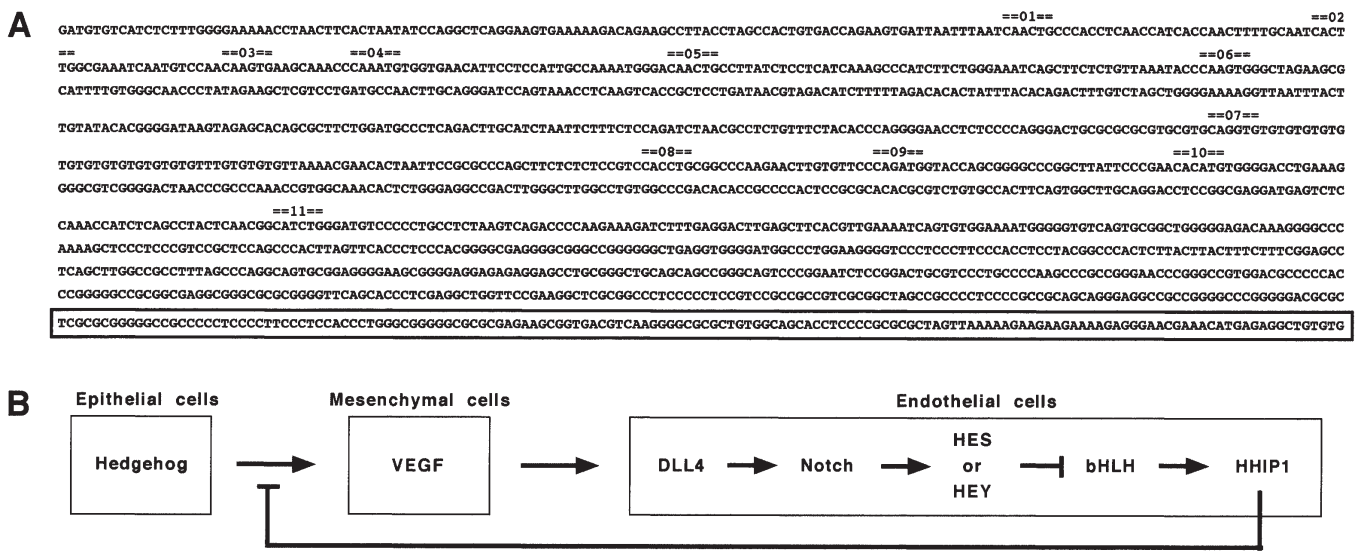

Figure 3. Regulation of *HHIP1* transcription in vascular endothelial cells. (A) Human *HHIP1* promoter. Exon 1 of human *HHIP1* gene is boxed. Eleven bHLH-binding sites within human *HHIP1* promoter are shown by double over-lines. (B) HHIP1 and Hedgehog-VEGF-Notch signaling cascade. Up-regulation of 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 down-regulation 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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