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A selective splicing variant of hepcidin mRNA in hepatocellular carcinoma cell lines

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ABSTRACT

Hepcidin is a main regulator of iron metabolism, of which abnormal expression affects intestinal absorption and reticuloendothelial sequestration of iron by interacting with ferroportin. It is also noted that abnormal iron accumulation is one of the key factors to facilitate promotion and progression of cancer including hepatoma. By RT-PCR/agarose gel electrophoresis of hepcidin mRNA in a hepatocellular carcinoma cell line HLF, a smaller mRNA band was shown in addition to the wild-type hepcidin mRNA. From sequencing analysis, this additional band was a selective splicing variant of hepcidin mRNA lacking exon 2 of *HAMP* gene, producing the transcript that encodes truncated peptide lacking 20 amino acids at the middle of preprohepcidin. In the present study, we used the digital PCR, because such a small amount of variant mRNA was difficult to quantitate by the conventional RT-PCR amplification. Among seven hepatoma-derived cell lines, six cell lines have significant copy numbers of this variant mRNA, but not in one cell line. In the transient transfection analysis of variant-type hepcidin cDNA, truncated preprohepcidin has a different character comparing with native preprohepcidin: its product is insensitive to digestion, and secreted into the medium as a whole preprohepcidin form without maturation. Loss or reduction of function of *HAMP* gene by aberrantly splicing may be a suitable phenomenon to obtain the proliferating advantage of hepatoma cells.

1. Introduction

Hepcidin is a central iron regulator derived from hepatocytes, of which function is to inhibit both enteric absorption and reticuloendothelial sequestration of iron through binding to ferroportin [1,2]. Its gene is called as *HAMP*, and composed of 3 exons and 2 introns in between, and the mature mRNA of hepcidin is formed by splicing [3]. As hepatocyte is a major organ of hepcidin production, the mature form of hepcidin, hepcidin-25 detected in serum is thought to reflect the status of body iron storage and inflammation [4]. Until now, there are couple of reports that the expression of hepcidin was altered in various types of diseases representing anemia of inflammation, although there were few studies investigating the expression of hepcidin in hepatocellular carcinoma (HCC). Studies using liver biopsy and surgical samples of HCC revealed that the expression of hepcidin mRNA was down-regulated in the tumorous tissues compared to the adjacent non-tumorous liver tissues [5–8]. Although the mechanism responsible for suppression of hepcidin mRNA expression remains unclear, this phenomenon may be advantageous for the growth of tumor cells, because duodenal enterocytes transfer iron to plasma is increased and resulted in an increase of body iron for cell growth by reduction of hepcidin. On the other hand, hepcidin mRNA

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expression is increased in response to inflammatory mediators such as IL-6, IL-1β and interferon α by invaded macrophages surrounding the tumors, which is the major cause of anemia of inflammation [9–11].

We previously demonstrated that there were differences in the expression of hepcidin mRNA among 12 kinds of cell lines derived from HCC by quantitative RT-PCR. Furthermore, there were unexpected differences in the secretion of hepcidin isoforms hepcidin-20, -22 and -25 among these HCC-derived cells by simultaneous quantification using liquid chromatography/selected reaction monitoring tandem mass spectrometry (LC-MS/MS) [12]. Concerning the mechanism of hepcidin regulation, a single nucleotide deletion of hepcidin mRNA, an elongated abnormal polypeptide, a new stop codon, and a decreases of transcriptional activity have been postulated [13–15]. However, up until now, the splicing variant of *HAMP* transcript have not been reported, of which form is common in transcripts of *HFE*, another regulatory molecule of iron metabolism [16].

By conventional RT-PCR/agarose gel electrophoresis for detecting a wild-type *HAMP* transcript, we found a new smaller hepcidin mRNA lacking an exon 2 of *HAMP* gene in HCC-derived cell line HLF. Then, by sensitive digital PCR using specific probes to this variant, we found that most of HCC-derived cell lines have significant copy numbers of hepcidin mRNA variant. Furthermore, we examined the molecular form secreted from cells by using the transient transfection analysis of variant-type hepcidin cDNA.

2. Materials and methods

2.1. Cell culture

293T was purchased from GenHunter Co. (Nashville, TN). HCC-derived cell lines, HepG2, Hep3B, HuH7, WRL68 and SK-HEP-1 were commercially obtained from DS Pharma Biomedical Co. (Osaka, Japan). HLE and HLF were supplied from the Health Science Research Resources Bank (Osaka, Japan).

2.2. First strand cDNA synthesis and polymerase chain reaction (RT-PCR)

RT-PCR was performed using PrimeScript™ RT and *TaKaRa Ex Taq*® HS (Takara Bio, Ohtsu, Japan). Primers specific from the start and the stop codon were used; forward primer: 5′-**ATG**GCACTGAGCTCCCAGAT-3′, reverse primer: 5′-**CTA**CGTCTTGCAGCACATCC-3’ (bold characters indicate start and stop codons, respectively).

2.3. Absolute quantification of hepcidin mRNA by digital PCR

For more sensitive and absolute quantification of hepcidin mRNA in cells, QuantStudio™ 3D Digital PCR System (Applied Biosystems®, Thermo Fisher Scientific, Waltham, MA) was used [17]. The PCR amplification was performed using a set of primers and the specific probe to variant-type hepcidin mRNA. First strand cDNA was synthesized using PrimeScript™ RT. PCR amplification was performed with QuantStudio™ 3D digital PCR master mix, on following cycle conditions: cycle 1, 96 °C for 10 min; cycle 2–39, 60 °C for 2 min, 98 °C for 30 s; and final step at 60 °C for 2 min, 10 °C infinitely.

2.4. Construction of preprohepcidin expression plasmids

Wild-type and variant-type preprohepcidin cDNA fragments were inserted into the mammalian expression plasmid, pCAcc [18]. Wild-type and variant-type preprohepcidin cDNA fragments without stop codon were cloned into the multi-cloning site of two tags containing plasmid, pcDNA™3.1(−)/myc-His/version A (invitrogen™, Thermo Fisher Scientific) in frame with these tags to create a fusion peptide in a C-terminus [19]. Each fragments of myc-His tagged preprohepcidin cDNA were separated and inserted back to the pCAcc plasmid.

2.5. Quantification of hepcidin isoform

293T cells were transfected with wild-type and variant-type preprohepcidin expression plasmids, respectively. 24 h and 48 h later, conditioned media were collected, and hepcidin isoforms were measured by LC-MS/MS as our previous report [12].

2.6. Western blot analysis

293T cells were transfected with pCAHAMP-myc-His, and pCA△HAMP-myc-His, respectively. 48 h later, conditioned media were collected, and these supernatants were incubated with 80 μl of Ni-NTA agarose suspension (Qiagen, Hilden, Germany) for 16 h at 4 °C. Pull-down polypeptides were eluted from Ni-NTA agarose by boiling for 5 min at 95 °C in 1 × Tricine sample buffer (Bio-Rad, Hercules, CA) with 2%(v/v) β-mercaptoethanol. Samples were separated by tricine-SDS/PAGE in 16.5% gels by the method of Schägger and von Jagow [20], and were transferred onto Immobilon-P ^{SQ} (Merck Millipore, Darmstadt, Germany). The blot was incubated with rabbit anti-myc tag antibody and anti-rabbit antibody conjugated with HRP, following with SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific).

3. Results

3.1. Identification of variant-type hepcidin mRNA

Hepcidin mRNA expression in a HCC-derived cell line, HLF was examined by using RT-PCR and agarose gel electrophoresis. The use of 2 primers complementary to the start and the stop codon regions was allowed the amplification of the predicted coding region of full length hepcidin mRNA. In electrophoresis, an amplicon of sourcing the full-length of preprohepcidin was detected (Fig. 1A). Interestingly, an additional band smaller than the full length size was obtained, suggesting that this amplicon was sourced from a variant formed mRNA. We then cloned and sequenced the corresponding two RT-PCR products. One was the correctly spliced full length transcript of 255 bp fragment (Fig. 1B). Another was the smaller one, which was identical to the wild-type preprohepcidin sequence except lacking of an internal 60 bp fragment in the NCBI Gene Reference NM_0221175.3 (Fig. 1C). We named this smaller fragment as Δ*HAMP*. In this alternative transcript, exon 2 is skipped without generating the frameshift and amino acid change in the new exon 1 − exon 3 junction.

3.2. Absolute quantification of variant-type hepcidin mRNA

In the present study, we used absolutely attainable chip-based digital PCR (digital PCR), because such small amount of variant-type hepcidin mRNA was difficult to quantitate in the conventional RT-PCR amplification. Furthermore, the quantitation of endpoint-amplicons separated on the agarose gel electrophoresis was not satisfactory [21]. From these reasons, to confirm this variant in HCC-derived cell lines, we performed the digital PCR to access the exact expression level of variant-type hepcidin mRNA by a specific probe.

To detect a wild-type hepcidin mRNA, the forward primer was designed to bind in the region of exon 1 and the reverse primer was designed to bind in the region of exon 2 (Fig. 2A). When wild-type

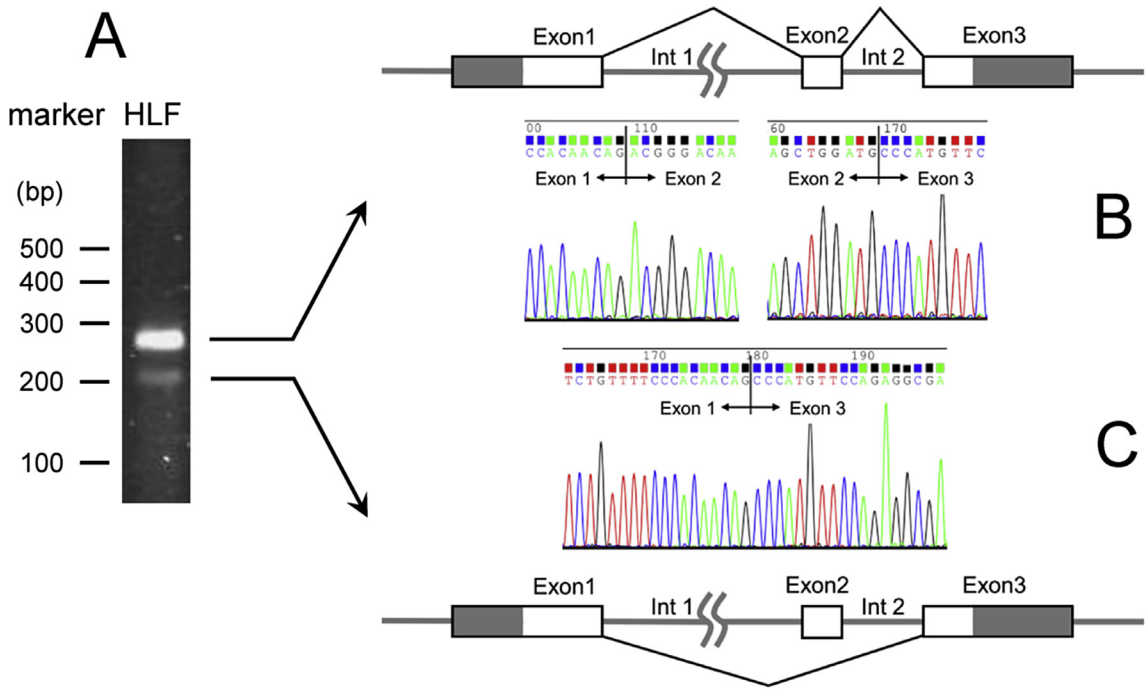


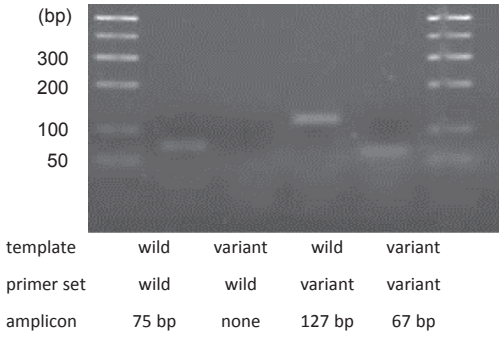
Fig. 1. Identification of an alternative splicing in the HAMP gene. (A) Expression of hepcidin mRNA in hepatocellular carcinoma cell line HLF was analyzed by RT-PCR, and products were run on 2% NuSieve™ GTG™ agarose gel/TAE. (B) Sequencing electrophoretogram of PCR product was shown each location of exon 1 – exon 2 and exon 2 – exon 3 junctions of wild-type hepcidin mRNA. (C) The location of a new exon 1 – exon 3 junction of variant-type hepcidin mRNA. Schematic organizations were indicated human *HAMP* gene and transcripts.

A

hepcidin mRNA (cDNA)	forward primer (Exon 1) 5'-TGACCAGTGGCTCTGTTTTCC-3'	reverse primer (Exon 2) 5'-TGGCTCCAGCTCTGTCCTG-3'	amplicon size	probe for wild-type (Exon 1 - 2 junction) 5'FAM-CACAACAGACGGGACAA-NFQ-MGB
wild-type	annealed	annealed	75 bp	hybridized
variant-type	annealed	not annealed	none	not hybridized

hepcidin mRNA (cDNA)	forward primer (Exon 1) 5'-TGACCAGTGGCTCTGTTTTCC-3'	reverse primer (Exon 3) 5'-GTGGGTGTCTCGCCTCCTT-3''	amplicon size	probe for variant-type (Exon 1 - 3 junction) 5'FAM-CAGCCCATGTTCCAG-NFQ-MGB
wild-type	annealed	annealed	127 bp	not hybridized
variant-type	annealed	annealed	67 bp	hybridized

B



C

cell line	hepcidin mRNA (copy number / μg RNA)	
	wild-type	variant-type
HepG2	66,036.6	538.1
Hep3B	93.6	0.0
HuH7	167,810.0	988.1
HLE	0.5	9.9
HLF	1.5	4.4
WRL68	2.8	31.2
SK-HEP-1	2.3	27.1

Fig. 2. Absolute quantification of hepcidin mRNA. (A) Primers and probes used in a chip-based digital PCR assays. (B) PCR amplifications were performed using each wild-type hepcidin cDNA and variant-type hepcidin cDNA as templates, and amplicons were resolved on agarose gel electrophoresis. (C) Absolute quantification of wild-type and variant-type hepcidin mRNAs in HCC-derived cell lines by digital PCR.

hepcidin mRNA was included in a sample, a single band of 75 bp in the size was amplified. On the other hand, an amplicon for variant-type hepcidin mRNA was not detected, because the reverse primer targeting to exon 2 could not anneal to variant-type hepcidin mRNA lacking the exon 2 region (Fig. 2B). To detect a variant-type hepcidin mRNA, the forward primer was designed to bind in the region of exon 1 and the reverse primer was designed to bind in the region of exon 3 (Fig. 2A). When variant-type hepcidin mRNA was included in a sample, a 67 bp band was amplified. In addition, the amplicon for wild-type hepcidin mRNA in the same sample was detected as a 127 bp band, because the reverse primer targeting to exon 3 can anneal to wild-type hepcidin mRNA, too (Fig. 2B). From these reasons, specific probes for the detection of each amplicon of wild-type and variant-type hepcidin mRNAs were necessary in this assay. These specific probes were designed for wild-type hepcidin mRNA which is bound at the original junction of exon 1 and exon 2, and for variant-type hepcidin mRNA which is bound at the new junction of exon 1 and exon 3, respectively (Fig. 2A). Fig. 2C shows the copy number/μg RNA of wild-type and variant-type hepcidin mRNAs. We detected variant-type hepcidin mRNA in 6 of 7 cell lines; HepG2 (538.1 copy numbers/μg RNA), HuH7 (988.1 copy numbers/μg RNA), HLE (9.9 copy numbers/μg RNA), HLF (4.4 copy numbers/μg RNA), WRL68 (31.2 copy numbers/μg RNA) and SK-HEP-1 (27.1 copy numbers/μg RNA), but not in Hep3B (0 copy number/μg RNA). In the assays of wild-type mRNA, two cell lines have higher expressions (66,036.6 copy numbers/μg RNA in HepG2 and 167,810.0 copy numbers/μg RNA in HuH7) and other five cell lines have lower expressions (93.6 copy numbers/μg RNA in Hep3B, 0.5 copy number/μg RNA in HLE, 1.5 copy numbers/μg RNA in HLF, 2.8 copy numbers/μg RNA in WRL68, and 2.3 copy numbers/μg RNA in SK-HEP-1).

3.3. Detection of mature hepcidin-25 translated from variant-type hepcidin mRNA

To evaluate whether mature hepcidin-25 is produced from variant-type hepcidin mRNA, transient transfection was conducted to collect the conditioned medium containing any secreted hepcidin. Schematic diagrams of the structure of hepcidin expression plasmids and expected polypeptides translated from each plasmid were shown in Fig. 3A and B.

The hepcidin-25 values in the conditioned medium from 293T cells transfected with wt *HAMP* cDNA were increased progressively from 53.6 ng/ml at 24 h to 193.9 ng/ml at 48 h. On the other hand, the hepcidin-25 values in the transfection with Δ*HAMP* cDNA were low; 2.3 ng/ml at 24 h and 4.1 ng/ml at 48 h, respectively (Fig. 3C). This result shows that mature hepcidin-25 is not generated efficiently from variant-type hepcidin mRNA. In addition, hepcidin isoforms; hepcidin-20 and -22 in the conditioned medium from 293T cells transfected with wt *HAMP* cDNA was detected, but not in the transfection of Δ*HAMP* cDNA (Fig. 3D).

3.4. Identification of secreted polypeptide form by variant-type hepcidin mRNA

To identify which polypeptides are secreted in the culture medium from variant-type hepcidin mRNA, transient transfection and pull-down assays were conducted. The expression plasmids of myc-His tagged preprohepcidin were schemed in Fig. 4A, and expected polypeptides from each myc-His tagged hepcidin cDNA were translated in Fig. 4B.

In the transfection of wt *HAMP* cDNA, in addition to the expected size length (12.3 kDa) of preprohepcidin-myc-His (triple arrowheads in Fig. 4C), two signals at 9.8 kDa (double arrowheads) and 5.7 kDa (single arrowhead) were detected in the spent medium; these predicted molecular weights correspond to prohepcidin-myc-His and hepcidin-25-myc-His, respectively. From this result, it was elucidated that the addition of myc-His tag to the C-terminal site of preprohepcidin had no influence in the posttranslational processes. On the other hand, in the transfection of a plasmid carrying Δ*HAMP* cDNA fused with the code for myc-His tag, a single band corresponding to the predicted molecular weight for truncated preprohepcidin-myc-His (10.1 kDa) was pull-downed from the spent medium (asterisk in Fig. 4C). Thus, most part of this truncated preprohepcidin-myc-His was secreted from the cell without posttranslational cleavage.

4. Discussion

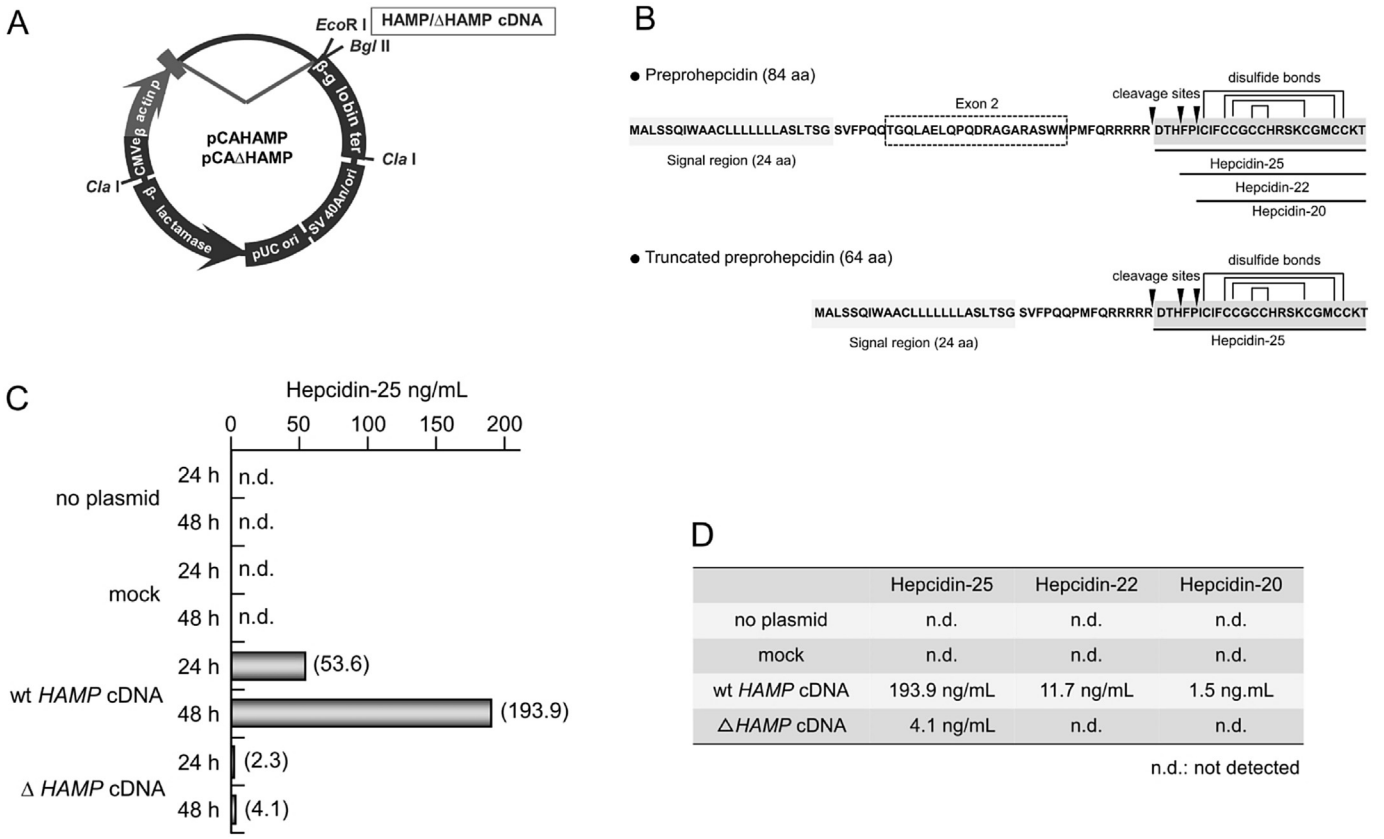
In this work, we found an alternative *HAMP* transcript in HCC-derived cell line, HLF, demonstrating that this transcript is a novel variant-type hepcidin mRNA encoding exon 2-lacked preprohepcidin. Furthermore, it was revealed that six of seven HCC-derived cell lines have a significant copy numbers of this variant by a digital PCR.

On the expression studies of hepcidin mRNA using liver biopsy and surgical samples of HCC revealed, it was generally down-regulated in the tumorous tissues compared to the adjacent non-tumorous liver tissues by using the pair of primers targeting hepcidin mRNA [5–8]. In two assays [5,6], the forward primer designed to exon 1 region of hepcidin mRNA, and the reverse primer to exon 3 region of that, and SYBR Green I inter-chelating reagent for detecting amplicons were used in real-time PCR. According to these primer structures, the level of hepcidin mRNA was estimated as a sum of hepcidin mRNA of wild-type mRNA and variant-type mRNA if any. In other two studies [7,8], the pair of primers targeting hepcidin mRNA were designed to exon 1–2 boundary and exon 3 regions respectively, the probe was only hybridized to wild-type hepcidin mRNA.

In the present study, we initially used the common primers located in exon 1 and exon 3, and we accidentally noticed the smaller band of abnormal splicing on one HCC-derived cell line HLF in agarose gel electrophoresis. By sequencing analysis of this smaller band, we found that this mRNA encodes the exon 2-lacked preprohepcidin. We therefore speculated that the conventional assay using these probes may miss the variant-type hepcidin mRNA. In order to dissolve these problems of hepcidin mRNA quantification, we concluded that specific probes of each amplicon of wild-type and variant-type hepcidin mRNAs are necessary for the detection in each assay. As shown in Fig. 2A, we constructed probes specific to wild-type and variant-type hepcidin mRNAs, respectively. In addition, a small amount of variant-type hepcidin mRNA was considered to be difficult to quantitate by the conventional RT-PCR amplification, and therefore the digital PCR was selected, which is more sensitive than the conventional RT-PCR amplification and enable to absolute quantification (copy numbers/μg RNA) in a sample [17].

Accordingly, six of seven HCC-derived cell lines; HepG2, HuH7, HLE, HLF, WRL68 and SK-HEP-1, but not Hep3B, expressed variant-type hepcidin mRNA between 4.4 and 988 copy numbers/μg RNA, while all the HCC-derived cell lines expressed wild-type hepcidin mRNA between 0.5 and 167,810 copy numbers/μg RNA. In the assays of wild-type mRNA, two cell lines have higher expressions and others have lower expressions, which may be consistent to previous reports that the expression of hepcidin mRNA was down-regulated in most of HCC [5–8].

Because human genes typically contain multiple introns, the process of pre-mRNA splicing is an essential step in the expression



D

	Hepcidin-25	Hepcidin-22	Hepcidin-20
no plasmid	n.d.	n.d.	n.d.
mock	n.d.	n.d.	n.d.
wt *HAMP* cDNA	193.9 ng/mL	11.7 ng/mL	1.5 ng.mL
Δ*HAMP* cDNA	4.1 ng/mL	n.d.	n.d.

n.d.: not detected

Fig. 3. Quantification of hepcidin isoforms secreted from cells. (A) Schematic diagram of a recombinant plasmid expressing preprohepcidin. (B) Amino acid sequences of preprohepcidin (adapted from reference no. 3), and truncated preprohepcidin which is removed the region from codon 31 to codon 50, respectively. (C) Conditioned media from which 293T cells were transiently transfected with *HAMP* cDNA, were collected and hepcidin-25 were measured by LC-MS/MS. no plasmid, non-transfected 293T cells; mock, original plasmid pCAcc; wt*HAMP* cDNA, full-length preprohepcidin cDNA (pCAHAMP); Δ*HAMP* cDNA, preprohepcidin cDNA lacked an internal 60 bases (pCAΔHAMP); n.d., not detected. (D) Quantification of hepcidin isoforms in conditioned media at 48 h after transfections.

of most genes [22]. Alternative patterns of intron removal allow the synthesis of multiple mRNAs encoding different proteins from a single gene [16]. The choice of alternative splicing pattern is often tissue specific, and can profoundly affect the activity of the encoded peptide [23]. An alternatively spliced exon is generally under the control of multiple splicing regulators [24]. In our analysis, any mutations, deletions and insertions were not found in three exons and two introns encompassing the full region of *HAMP* gene (NCBI Reference Sequence: NG_011563.1) in HLF cell line. This splicing variant does not fit with any mechanism resulting from known splicing consensus sequences [25]. In addition, variant-type hepcidin mRNA might not be subjected to nonsense-mediated decay (NMD) that is believed to be a mechanism for proofreading of transcripts to prevent the inappropriate expression of aberrant proteins [26]. Therefore, we speculated that variant-type hepcidin mRNA escaped from NMD. Furthermore, whole-exome sequencings revealed that any predominant mutation on the component genes of the RNA splicing machinery was not found in the all seven cell lines.

To clarify the characteristic features of a new peptide from variant-type hepcidin mRNA, we used the transient transfection to 293T cells, in which there was no detectable level of *HAMP* transcripts and mature hepcidin-25 under the conventional culture condition. In the western blot analysis of pull-down-extracts from the spent medium, we detected three bands corresponding to the predicted molecular weights for preprohepcidin-myc-His, prohepcidin-myc-His and hepcidin-25-myc-His, respectively. In other words, when 293T cells were transfected with the expression plasmid of myc-His tagged wild-type preprohepcidin, the myc-His tagged preprohepcidin produced in 293T cells was correctly processed. Nevertheless, from spent medium of 293T cells overexpressed with the truncated preprohepcidin-myc-His, most part of peptides was secreted from cells without the posttranslational processing. In addition, when 293T cells were transfected with the expression plasmid of tag-free truncated preprohepcidin, the level of mature hepcidin-25 in the culture medium was very low comparing to that in wild-type preprohepcidin transfection. From these results, 20 amino acids residues comprising exon 2 in the *HAMP* gene which are located between the secretory signal peptide and the motif peptide of proprotein convertase, will be regarded as structural residues that participate in maintaining the scaffold which only limited enzymes can attach and work. In contrast to normal cells, aberrantly splicing variants may lead to loss or reduction of function of these genes in cancer, and these are suitable phenomena for the growth of cancer cells [27,28].

In summary, our paper is the first to demonstrate that there was an aberrant splicing from of the *HAMP* gene, producing the transcript that encodes truncated peptide lacking 20 amino acids at the middle of preprohepcidin. This alternative transcript results in a 64 aa truncated preprohepcidin: its product is insensitive to digestion, and secreted from cells as a whole molecule form without maturation. Further work is required to identify the physiological and/or pathological situations where variant-type hepcidin mRNA might be expressed, and to evaluate its role.

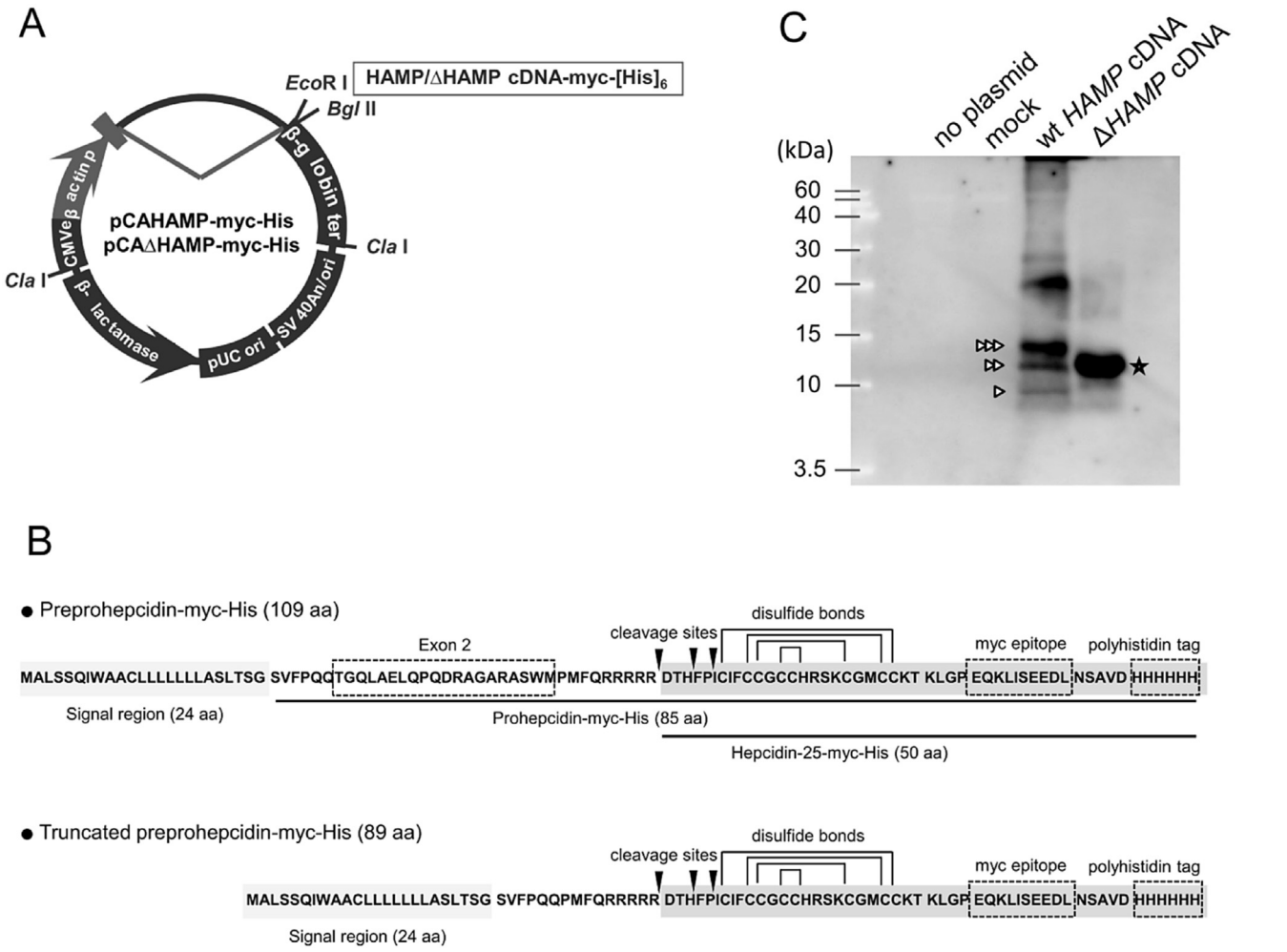


Fig. 4. Analysis of polypeptides secreted from cells. (A) Schematic diagram of a recombinant plasmid expressing myc-His tagged preprohepcidin. (B) Amino acid sequences of myc-His tagged preprohepcidin and truncated preprohepcidin, respectively. (C) Conditioned media from which 293T cells were transiently transfected with *HAMP* cDNA, were collected and secreted polypeptides tagged with myc-His were analyzed by Western blotting. no plasmid, non-transfected 293T cells; mock, original plasmid pCAcc; wt*HAMP* cDNA, full-length myc-His tagged preprohepcidin cDNA (pCAHAMP-myc-His); Δ*HAMP* cDNA, myc-His tagged preprohepcidin cDNA lacked an internal 60 bases (pCAΔHAMP-myc-His); n.d., not detected. ▷▷▷, preprohepcidin-myc-His; ▷▷, prohepcidin-myc-His; ▷, hepcidin-25-myc-His; ☆, truncated preprohepcidin-myc-His.

Conflict of interest

K.S. and Y.K have received research funding from Novartis Pharmaceuticals Japan Co., Ltd. (Tokyo, Japan). The remaining authors declare no competing financial interests. The Department of Gastrointestinal Immunology and Regenerative Medicine is endowed by KyorinPhamaceutical Co., Ltd. (Tokyo, Japan), Sapporo Higashi Tokushukai Hospital (Sapporo, Japan), Asahi Kasei Medical Co., Ltd. (Tokyo, Japan), and Novartis Pharmaceuticals Japan Co., Ltd. (Tokyo, Japan). Quantification of hepcidin isoforms in cell cultures was performed in collaboration with Chugai Pharmaceuticals Japan Co., Ltd. (Tokyo, Japan).

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