Iron Metabolism • Research Paper

Hepatic and extrahepatic expression of the new iron regulatory protein hemojuvelin

[haematologica]
2004;89:1441-1445

ALEJANDRA RODRIGUEZ MARTINEZ
ONNI NIEMELÄ
SEPPO PARKKILA

A B S T R A C T

Background and Objectives. Hereditary hemochromatosis (HH) is a common disorder of iron overload. A rare variant of the disease, juvenile hemochromatosis, is an early-onset form which is caused by mutations in a recently identified gene, called *HJV* or *HFE2*. A previous report based on Northern blotting showed human HJV mRNA expression only in the skeletal muscle, liver and heart.

Design and Methods. In this study we analyzed the expression of HJV mRNA in a number of human and mouse tissues by a sensitive reverse transcription-polymerase chain reaction method. We also studied the expression of HJV protein in mouse tissues using Western blotting. A polyclonal rabbit antibody was raised against a synthetic peptide which was designed based on the predicted sequence of human and mouse HJV protein.

Results. Human HJV mRNA expression was detected in the liver, heart, esophagus, pancreas, descending colon, ileocecum and skeletal muscle. Mouse tissues that were positive for expression included brain, liver, heart, lung, stomach, spleen, kidney, duodenum, jejunum, ileum, colon, skeletal muscle, testis and blood. By Western blotting, HJV protein expression was detected in the mouse liver, heart, kidney, brain and muscle.

Interpretation and Conclusions. The facts that HJV protein is expressed in the liver and mutations in the *HJV* gene induce hepatic iron accumulation point to a possibility that HJV protein may modulate iron transport in hepatocytes. The wide expression of HJV as shown in the present study suggests that its role in regulating iron allocation could be extended to other tissues beyond the liver.

Key words: expression, hemochromatosis, hemojuvelin, *HFE2*, mRNA.

From the Institute of Medical Technology (ARM, SP) and Department of Clinical Chemistry, University of Tampere and Tampere University Hospital, Tampere, Finland (ARM, ON, SP); Laboratory of EP Central Hospital, Seinäjoki, Finland (ON); Department of Clinical Chemistry, University of Oulu, Oulu, Finland (SP).

Correspondence:
Prof. Seppo Parkkila, MD, PhD, Institute of Medical Technology, University of Tampere, Biokatu 6, FI-33520 Tampere, Finland.
E-mail: seppo.parkkila@uta.fi

Hereditary hemochromatosis (HH) consists of a group of genetic disorders which lead to iron deposition in several tissues and consequent damage of organs, such as the liver, heart, and pancreas. Several variant forms of HH have been described with heterogeneous etiology and slight clinical differences (Table 1). In the classical form of HH (type 1), late complications in the absence of treatment usually appear between the 4th and the 5th decades of life. The medical problems manifested in HH include cirrhosis, hepatocellular carcinoma, cardiomyopathy, diabetes mellitus and hypogonadism. Type 1 HH is associated with mutations within the *HFE* gene.[1-3] Nevertheless, HH is genetically a heterogeneous disease, and the penetrance of different genotypes has remained unknown.[4] The HFE protein appears to have direct functional links to iron transport, since it was found to associate and interact with transferrin receptor (TfR) in cryptal enterocytes and in placental syncytiotrophoblasts.[5-7] In these locations, HFE protein is believed to have an important role in TfR-mediated iron transport. The ultimate proof that deficiency or functional derangement of the *HFE* gene product is the molecular basis of HH was provided when *HFE* gene knockout mice were found to exhibit hepatic iron accumulation in a manner similar to that in the classical form of HH.[8]

In juvenile hemochromatosis (JH), also called type 2 hemochromatosis, iron overload occurs at a young age, leading to clinical symptoms usually before 30 years of age. Severe and early development of cardiomyopathy and hypogonadism are characteristic features of this disorder, which affects both sexes equally.[9] Mutations in the hepcidin gene have been reported in some JH

Table 1. Background information on hereditary hemochromatosis (HH).

Genes of iron metabolism		*Types of HH*	
Gene/protein	*OMIM*	*Name of disorder*	*locus*
HFE/HFE	235200	Type 1, HFE-related HH	6p21.3
HFE2, HJV/Hemojuvelin	608374	Type 2A, juvenile hemochromatosis	1q21
HAMP/Hepcidin	606464	Type 2B, juvenile hemochromatosis	19q13
TFR2/Transferin receptor-2	604720	Type 3	7q22

patients.[10] Hepcidin is a small disulfide-bonded peptide with antimicrobial activity which is expressed primarily in the liver.[11,12] However, several reports demonstrated linkage to a locus on chromosome 1q21 in the majority of JH cases.[13-16] This locus was recently shown to contain a gene, called *HJV* or *HFE2*, which is probably the main causative gene of JH.[17] In the original report, Papanikolaou *et al.*[17] identified four missense mutations, one nonsense mutation and one frameshift mutation from 12 patients. In a subsequent report, Lee *et al.*[18] described two other mutations in the same gene. The *HJV* gene is 4,265 bp long and contains four exons.[17] It is transcribed into a full-length messenger-RNA with five spliced isoforms. Hemojuvelin (HJV), the protein product of the *HJV* gene, exists in three isoforms of 426, 313 and 200 amino acids. The possible function of HJV protein as a membrane-bound receptor or secreted polypeptide hormone was hypothesized based on several protein motifs identified in its amino acid sequence. Even though the exact function of HJV protein has not been established it is believed to have a role in iron metabolism.

A previous report based on Northern blotting analysis showed human HJV mRNA expression in skeletal muscle, liver and heart.[17] The present study was designed to investigate further the expression of HJV mRNA in human and mouse tissues as well as to provide the first evidence on HJV protein expression in mouse tissues.

Design and Methods

Polymerase chain reaction (PCR) analysis

The expression of human and mouse HJV mRNA was first examined using cDNA kits purchased from BD Biosciences (Palo Alto, CA, USA). The cDNA included in MTC™ panels were used as templates for PCR using *HJV* gene-specific primers. The human MTC™ digestive panel, panel I and panel II and mouse MTC™ panel I (BD Biosciences) contained first-strand cDNA preparations produced from total poly(A) RNA isolated from a number of different tissues. All human cDNA were derived from adult tissues. Another set of mouse cDNA was prepared in our laboratory. Total RNA from mouse tissue samples (pooled from 5 adult Balb/c mice) was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA using M-MuLV reverse transcriptase (Finnzymes, Espoo, Finland) using random primers (500 μg/mL) according to the manufacturer's instructions.

Two primers for amplifying human HJV cDNA were chosen based on the human mRNA sequence (GenBank database accession No. AY372521) which is the translated portion of the gene's longest transcript; forward 5′-TCACTTTCACACATGCCG-3' (nucleotides 540-557 in exon 3) and reverse 5'-GATCGAGAGAGTCGCTGAC-3' (nucleotides 971-989 in exon 4), which generated a 450-bp product corresponding to amino acids 180-330 of the protein sequence. The primers were produced by Sigma Genosys (Cambridgeshire, UK). Primers for β2-microglobulin were used to monitor the quantity of mRNA in the study samples.

To amplify mouse HJV cDNA, two primers (Sigma Genosys) were chosen based on the mouse HJV mRNA sequence published in GenBank (NM_027126); forward 5'-AGGCTGAGGTGGACAATC-3' (nucleotides 945-962) and reverse 5'-CAAGAAGACTCGGGCATC-3' (nucleotides 1382-1399), which generated a 455-bp product corresponding to amino acids 230-381 of the murine HJV protein sequence.[17] Primers for β-actin were used to assess the quantity of mRNA.

Amplification was performed using 2-3 ng of total cDNA as a template. The PCR reactions were amplified in a thermal cycler (Biometra, Göttingen, Germany). After initial denaturation at 94°C for 1 min, amplification was performed for 30-31 cycles of denaturation at 94°C for 30 s, annealing at 55-56°C for 30 s and extension at 72°C for 1 min 30 s, followed by a final extension at 72°C for 3 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel containing 0.1 μg/mL ethidium bromide with DNA standard (100 bp DNA Ladder, New England Biolabs, Beverly, MA, USA). Primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH, BD Biosciences) were used in all the performed experiments to monitor the amplification reaction.

The amplification products obtained from the human liver and muscle and mouse blood and 17-day old

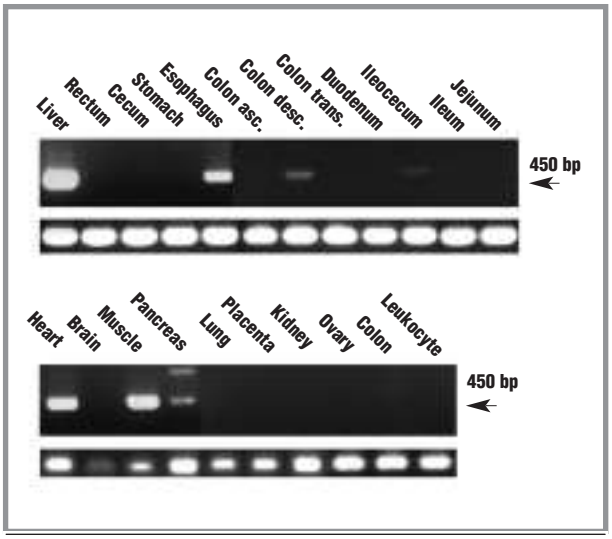

Figure 1. PCR analysis of human HJV mRNA expression. The amplified sequence partially includes exons 3 and 4. The strongest 450 bp signals are seen in the liver, esophagus, heart and muscle. Weaker bands are present in the descending colon and pancreas. A faint signal is present in the ileocecum. β-2-microglobulin primers were used to monitor the quantity of mRNA in the samples.

embryos were purified from agarose gel using GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences, Buckinghamshire, UK). DNA sequencing reactions were performed using ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. The sequencing was finally performed on the ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems).

Antibodies

The rabbit anti-human/mouse HJV serum was raised by Innovagen AB (Lund, Sweden) against a synthetic peptide (NH_2-) SRSERNRRGAITIDTARRLC (-COOH) which was designed based on the predicted amino acid sequences of human and mouse HJV (amino acids 328-347 and 321-340 from human and murine HJV molecules, respectively).[17] Both mouse and human HJV mRNA sequences are available in GenBank (accession numbers AY372521 and NM_027126).

Western blotting

Samples of heart, lung, stomach, duodenum, jejunum, liver, ileum, colon, spleen, kidney, muscle, testis and brain were obtained from adult Balb/c mice. The procedures were approved by the institutional animal care committee (University of Tampere). The tissue samples were homogenized in phosphate-buffered saline (PBS) in the presence of protease inhibitors, and approximately 1 mg of protein from each sample was analyzed by SDS-PAGE (NuPAGE 10% Bis-Tris, Invitrogen) under reducing conditions according to Laemmli.[19] The separated proteins were transferred electrophoretically from the gel to a Parablot polyvinylidene fluoride membrane (Macherey-Nagel, Düren, Germany) in a Novex Xcell II blot module (Invitrogen). After the transblotting, HJV protein was detected by the electroluminescence method (Amersham Biosciences) according to the manufacturer's instructions. The primary antibody was diluted 1:2000. Control experiments were performed using preimmune serum (1:1000) instead of the anti-HJV serum.

Results

HJV gene expression in human tissues

The expression of the human *HJV* gene was studied by PCR amplification of a commercially available set of cDNA produced from selected human tissues, including liver, rectum, cecum, stomach, esophagus, ascending colon, descending colon, transverse colon, duodenum, ileocecum, ileum, jejunum, heart, brain, muscle, pancreas, lung, placenta, kidney, ovary, colon and leukocytes. Figure 1 shows a strong 450-bp band in the liver, esophagus, heart and muscle and weaker reactions in the descending colon and pancreas. A faint signal was also observed in the ileocecum. Rectum, cecum, stomach, ascending colon, transverse colon, duodenum, ileum, jejunum, brain, lung, placenta, kidney, ovary, colon and leukocytes were negative.

HJV gene expression in mouse tissues

The expression of the murine *HJV* gene was investigated by PCR amplification of cDNA samples produced in our laboratory out of 5 Balb/c mice (including blood, heart, lung, stomach, duodenum, jejunum, liver, ileum, colon, spleen, kidney, muscle, testis and brain) (Figure 2, upper panel). In addition, we used a commercially available set of cDNA produced from selected mouse specimens (including heart, brain, spleen, lung, testis, 7-day old embryo, 11-day old embryo, 15-day old embryo and 17-day old embryo) (Figure 2, lower panel). Positive 455-bp bands were observed in all tissue samples except for the brain and spleen of the commercial panel. It is of interest that a strong positive signal was amplified from the brain and spleen cDNA prepared in our laboratory. The three oldest embryos showed positive bands. Even though the PCR method was not quantitative, the signal became stronger with increasing age of the embryo, which could indicate a developmental regulation.

Expression of hemojuvelin in mouse tissues

Expression of mouse HJV protein was analyzed using Western blotting. Compared to the results in RT-PCR, the

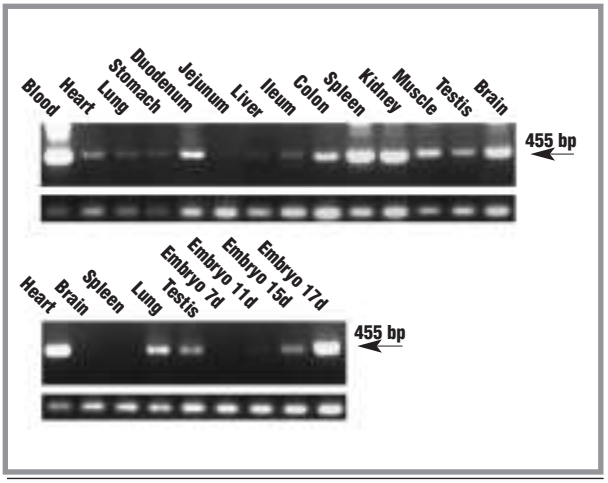

Figure 2. Expression of HJV mRNA in mouse tissues. The upper panel shows the results derived from the cDNA samples produced in our laboratory. The lower panel represents the commercial cDNA samples. Positive bands are observed in most tissue specimens except the brain and spleen in the lower panel. Note that the bands in the embryo samples become stronger with age. β-actin primers were used as an internal control to assess the quantity of mRNA.

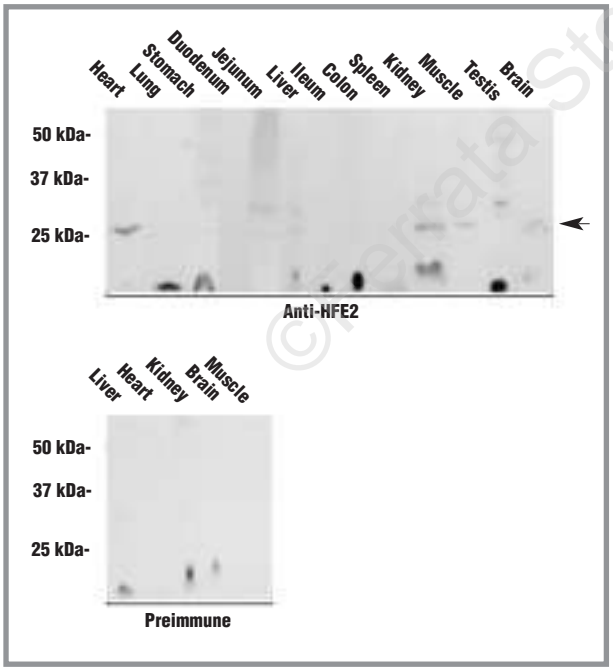

Figure 3. Western blotting of HJV protein in mouse tissues. The antibody was raised against a synthetic peptide which was designed based on the predicted amino acid sequences of human and mouse HJV. A positive polypeptide band of 26 kDa is present in the samples from heart, liver, kidney, muscle and brain. A band of 30 kDa is also observed in the samples from jejunum, liver, kidney and testis.

protein expression showed a more limited pattern of distribution. The results are shown in Figure 3. A 26 kDa positive band was observed in the liver, heart, kidney, brain and muscle. A 30 kDa band was also observed in the jejunum, liver, kidney and testis. These two bands most likely correspond to different HJV isoforms.[17] Both the antiserum and pre-immune serum showed low molecular weight bands in several lanes. These non-specific bands are probably present because of the high protein content in each lane.

Discussion

The present study was designed to elucidate HJV protein and mRNA expression in different human and mouse tissues. The RT-PCR results for human HJV agreed quite well with the distribution pattern recently reported by Papanikolaou *et al.*[17] In addition to the previously documented sites of expression (skeletal muscle, liver and heart), we found positive signals in four other tissues: descending colon, esophagus, ileocecum and pancreas. Of these, the last three were not previously tested for HJV expression.[17] The RT-PCR results indicated positive expression only in the descending colon, whereas the other segments of the large intestine were negative. Therefore, it is not surprising that the Northern blot result for colon has previously remained negative.[17] The observed variation may simply reflect different sampling. It is also notable that the signal intensities of RT-PCR and Northern blot did not correlate with each other.[17] This discrepancy can be attributed to the nature of the different methods. The RT-PCR method employed in the present study is not quantitative, although it is considered more sensitive than the Northern blotting.

In this paper, we also report the first results on HJV expression in mouse tissues. The data indicate that the transcript is more widely expressed than its human ortholog. In fact, the mRNA seems to be present in nearly every mouse tissue tested. This difference in the distribution of HJV mRNA may point to a physiological interspecies variation of iron metabolism. On the other hand, it is possible that the heterogeneity of primers could affect the results in the sensitive RT-PCR method. To confirm the specificity of the PCR reactions, the amplification products obtained from the human liver and muscle and mouse blood and 17-day old embryo were purified from agarose gel and subjected to DNA sequencing. The correct product was amplified from each tissue (*data not shown*). Even though we must be careful with attributing a quantification relevance to RT-PCR results, it is noteworthy that the *HJV* transcript seems to emerge during embryogenesis. No signal was visible at embryonic day 7, a positive band became

apparent in 11-day old embryos, and was found to further increase during the later stages. Absence of the specific mRNA during the early stages of development could indicate that HJV protein is not required during early processes such as blastocyst formation and implantation. However, the transcript was present at 11 days, which represents the peak time for organogenesis in mice. Based on those preliminary results, it would be interesting to correlate HJV expression to the developmental stages in more detailed investigations using *in situ* hybridization or immunohistochemistry.

Figure 2 showed some heterogeneity in HJV mRNA expression between different mice. Positive HJV signals were amplified from all cDNA samples, which were produced in our laboratory. In contrast, no amplification product was obtained from the commercial brain and spleen cDNA. The former cDNA were pooled from 5 mice and the latter cDNA from 200 mice. Thus, it is unlikely that any coincidences could explain this variation. Previous studies have demonstrated the influence of mouse strain on the severity of iron accumulation.[20, 21] Therefore, one can hypothesize that the expression of iron regulatory proteins including HJV could be affected by genetic factors linked to different strains or mouse colonies. Therefore, further studies should be conducted to evaluate HJV expression in several mouse strains as well as in mice with defective regulation of iron metabolism. According to our observations mouse HJV mRNA is more widely expressed than the corresponding protein. The differences in the distribution could be due to a higher sensitivity of the PCR amplification method as compared to the immunodetection. Another possibility is that the more limited protein expression pattern derives from post-transcriptional regulation. Nevertheless, the data suggests that HJV mRNA is expressed in a number of different human and mouse tissues. Thus, the role of the HJV protein may not be restricted to those organs, which are classically considered most important for orchestrating iron allocation. Mouse HJV protein was also detected in tissues where iron is primarily accumulated during the development of juvenile hemochromatosis, which supports a role for the HJV protein in the regulation of iron transport in these tissues. Although as yet only little is known about this interesting protein, the present results should open avenues for cell level-oriented studies on the role and mechanisms of HJV protein in the regulation of iron homeostasis.

All authors (ARM, ON, SP) gave substantial contributions to the conception and design of the study, analysis and interpretation of data, drafting and revising the article critically, and gave the final approval of the present version of the manuscript. ARM performed most of the laboratory analyses. SP and ON supervised the study.

This work was supported by grants from the Sigrid Juselius Foundation and Academy of Finland to SP, and from the Finnish Foundation for Alcohol Studies to ON.

The authors reported no potential conflicts of interest.

Manucript received June 22, 2004. Accepted September 6, 2004.

References

1. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 1996; 13:399-408.
2. Trinder D, Fox C, Vautier G, Olynyk JK. Molecular pathogenesis of iron overload. Gut 2002;51:290-5.
3. Parkkila S, Niemela O, Britton RS, Fleming RE, Waheed A, Bacon BR, et al. Molecular aspects of iron absorption and HFE expression. Gastroenterology 2001;121:1489-96.
4. Merryweather-Clarke AT, Cadet E, Bomford A, Capron D, Viprakasit V, Miller A, et al. Digenic inheritance of mutations in HAMP and HFE results in different types of haemochromatosis. Hum Mol Genet 2003;12:2241-7.
5. Lebron JA, Bennett MJ, Vaughn DE, Chirino AJ, Snow PM, Mintier GA, et al. Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. Cell 1998;93:111-23.
6. Parkkila S, Waheed A, Britton RS, Bacon BR, Zhou XY, Tomatsu S, et al. Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. Proc Natl Acad Sci USA 1997;94:13198-202.
7. Waheed A, Parkkila S, Saarnio J, Fleming RE, Zhou XY, Tomatsu S, et al. Association of HFE protein with transferrin receptor in crypt enterocytes of human duodenum. Proc Natl Acad Sci USA 1999;96:1579-84.
8. Zhou XY, Tomatsu S, Fleming RE, Parkkila S, Waheed A, Jiang J, et al. HFE gene knockout produces mouse model of hereditary hemochromatosis. Proc Natl Acad Sci USA 1998; 95:2492-7.
9. De Gobbi M, Roetto A, Piperno A, Mariani R, Alberti F, Papanikolaou G, et al. Natural history of juvenile haemochromatosis. Br J Haematol 2002;117:973-9.
10. Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, et al. Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet 2003;33:21-2.
11. Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147-50.
12. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001; 276: 7806-10.
13. Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'Ascola G, et al. Juvenile hemochromatosis locus maps to chromosome 1q. Am J Hum Genet 1999;64:1388-93.
14. Papanikolaou G, Politou M, Roetto A, Bosio S, Sakelaropoulos N, Camaschella C, et al. Linkage to chromosome 1q in Greek families with juvenile hemochromatosis. Blood Cells Mol Dis 2001;27:744-9.
15. Barton JC, Rao SV, Pereira NM, Gelbart T, Beutler E, Rivers CA, et al. Juvenile hemochromatosis in the southeastern United States: a report of seven cases in two kinships. Blood Cells Mol Dis 2002; 29:104-15.
16. Rivard SR, Lanzara C, Grimard D, Carella M, Simard H, Ficarella R, et al. Juvenile hemochromatosis locus maps to chromosome 1q in a French Canadian population. Eur J Hum Genet 2003;11:585-9.
17. Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dube MP, et al. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet 2004;36:77-82.
18. Lee PL, Beutler E, Rao SV, Barton JC. Genetic abnormalities and juvenile hemochromatosis: mutations of the HJV gene encoding hemojuvelin. Blood 2004;103:4669-71.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
20. Fleming RE, Holden CC, Tomatsu S, Waheed A, Brunt EM, Britton RS, et al. Mouse strain differences determine severity of iron accumulation in Hfe knockout model of hereditary hemochromatosis. Proc Natl Acad Sci USA 2001;98:2707-11.
21. Leboeuf RC, Tolson D, Heinecke JW. Dissociation between tissue iron concentrations and transferrin saturation among inbred mouse strains. J Lab Clin Med 1995;126:128-36.