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Upstream stimulatory factor 2 is implicated in the progression of biliary atresia by regulation of hepcidin expression☆

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Abstract

Background: Hepcidin is downregulated during the progression of biliary atresia (BA), but the mechanism is still unknown.

Methods: We analyzed single nucleotide polymorphism of rs7251432 and 916145 within hepcidin and its upstream, USF2 gene, respectively, in 52 patients of BA and 96 healthy controls. Liver tissues were obtained from 10 patients with early and late stage of BA, 10 patients with choledochal cyst, and 4 normal controls to study upstream stimulatory factor 2 (USF2) messenger RNA (mRNA) and protein expressions. Chromatin immunoprecipitation assay and USF2-specific short interference RNA (siRNA) were used in human HepG2 cells to show that USF2 can regulate hepcidin expression.

Results: C and CC allele frequencies of rs916145 of USF2 were significantly higher in patients with BA than in healthy controls. There was also significantly higher USF2 protein nuclear translocation in the early stage of BA than in the late stage, which was compatible with higher hepcidin mRNA expression in the early stage of BA. Chromatin immunoprecipitation assay demonstrated physiologic bindings of USF2 to the hepcidin promoter in HepG2 cells. USF2 siRNA also significantly knocked down hepcidin mRNA expression.

Conclusion: The study demonstrates that C allele of rs916145 in USF2 gene has more frequency for developing BA, and decreased USF2 protein nuclear translocation might partly play a role in the decreased hepcidin expression in the cholestatic liver injury of the late stage of BA.

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Biliary atresia (BA) is characterized by progressive destruction and complete obliteration of the extrahepatic bile ducts within months of birth and is very much unknown as far as etiology is concerned. Some infectious pathogens, particularly the virus, have been identified in limited number of patients with BA, but none is universally identified in every case [1,2]. Genetic factors have been implicated in the pathogenesis, which is supported by the presence of congenital nonhepatic malformations and by association of single nucleotide polymorphism (SNP) at CD14/−159 with development of BA and idiopathic neonatal cholestasis [3-5]. Decreased soluble CD14 was found in T/T and T/C genotype of BA in those patients prone to develop liver cirrhosis after Kasai procedure (KP) [5]. Arikan et al [6] also found that −173C allele of the macrophage migration inhibitory factor gene might be associated with the susceptibility to BA.

In our previous study, hepatic expression of hepcidin in BA was unique in that it decreased remarkably in the cirrhotic liver because of BA but not because of hepatitis B when compared with the early stage of BA and corresponding adult controls, respectively [7]. Moreover, plasma hepcidin levels were higher in jaundice-free patients with BA post-KP than patients with failed KP who needed liver transplantation (LT). Human hepcidin was first isolated and characterized as a highly disulfide-bonded peptide with antimicrobial activity, mainly expressed in liver, and was also named liver-expressed antimicrobial peptide 1, or LEAP-1 [8]. Moreover, hepcidin, a gene that is upregulated in the liver by inflammation and iron overload, is a negative regulator of iron absorption from the duodenum and is released from macrophages [9-12]. Hepcidin has a significant antimicrobial property for *Escherichia coli* [13], which is the most common bacterium involved in postoperative cholangitis in BA [14], which exacerbates preexisting liver fibrosis, leading to end-stage liver cirrhosis. In the study of vitality of tilapia, *Oreochromis mossambicus*, Huang et al [15] found that these fishes had high expression of hepcidin, and they proposed that hepcidin played important roles in resisting pathogenic infection. It seemed that higher hepcidin has a protection role during the progression of BA at least, in part, in preventing from repeat cholangitis from *E. coli* infection.

The control of hepcidin expression is very complex, which includes interaction of inflammatory cytokine, predominantly interleukin 6 with signal transducers and activators of transcription protein (STAT3), iron metabolism through bone morphogenetic protein (BMP)/Smad4 pathway, and reactive oxygen species through CCAAT/enhancer-binding protein α (C/EBPα) [16-19]. In a study of upstream stimulatory factor 2 (USF2) knockout mice, Nicolas et al [20] incidentally found that the mice presented with hemochromatosis were devoid of hepcidin expression. USF2 lies just upstream to hepcidin gene and serves as *cis* and *trans* regulation of hepcidin expression [21]. In addition to regulating glucose metabolism, cellular proliferation, and growth, USF2 can also regulate C/EBPα gene expression during differentiation [22,23]. Taking together, it is meaningful to study genetic predisposition to BA by investigating the common SNPs within hepcidin and its upstream regulatory USF2 gene, and to correlate USF2 expression with hepcidin expression during the progression of BA, and to study USF2 regulation of hepcidin expression in HepG2 cell.

1. Methods

1.1. Patients and samples

Genomic DNA of 96 anonymous unrelated healthy controls (54 male and 42 female) served as normal controls, and 52 with established diagnosis of BA (25 male and 27 female) were obtained either from buffy coat leukocytes by the standard phenol/chloroform extraction method or from the archival liver specimens by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Fresh liver tissues were obtained from 4 male and 6 female patients at the early stage of BA, when they received KP, and 3 male and 7 female patients at the late stage of BA when they received LT. Ten liver samples, which served as disease controls, were from 4 male and 6 female patients with choledochal cyst when they underwent surgical intervention. The rationale to use choledochal cyst as a disease control is that the etiology is more likely to be congenital than of infectious origin [24]. Four normal control liver samples were from 2 male and 2 female patients undergoing liver resection for benign lesion of various causes in which the livers were devoid of fibrosis and cholestasis. Detailed history of the patients was recorded, including age when the patient received operation, sex, and serum aspartate aminotransferase, alanine aminotransferase, and total bilirubin (Table 1).The study was approved by the Ethics and Clinical Research Committee of the Chang Gung Memorial Hospital, Taiwan, ROC, and informed consent had been obtained from the parents.

1.2. Detection of SNP

An SNP is the most common type of genetic variation. It is expected that SNPs will allow us to look for associations between a disease and specific sequence differences in a population of individuals. Reference SNP(rs) number can be archived from National Center for Biotechnology Information database of genetic variation (dbSNP). We analyzed the most commonly assayed SNP of rs7251432 and 916145 within the intron of hepcidin and USF2 gene, respectively, by using a commercially available SNAPSHOT kit (Applied Biosystems, Foster City, CA). We chose these 2 SNPs because their allelic distribution have been validated in different ethnic groups. SNPs were genotyped with TaqMan technology (Assay-by-Design) on the ABI7700 instrument (Applied Biosystems, Foster City, CA). All probes and