The Significance of Human Jagged 1 Mutations Detected in Severe Cases of Extrahepatic Biliary Atresia

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Mutations of human jagged 1 (JAG1) gene are responsible for Alagille Syndrome (AGS), whose 2 main symptoms are intrahepatic bile duct hypoplasia and pulmonary stenosis. We examined the JAG1 mutation in extrahepatic biliary atresia (EHBA), which is similar in phenotype to AGS, although a different pathogenesis is suggested. In 102 cases of EHBA, 9 missense mutations were detected, including 2 intrafamilial expressions in the propositus and an aunt of one family. These mutations were all missense and sporadic except for those of this particular family. The JAG1 gene mutations were generally found in severely ill patients subjected to liver transplantation at less than 5 years of age. None of the 9 cases of EHBA revealed any of the 5 major symptoms of AGS nor any identical pathological findings after 3 years of follow-up. Our cases were clearly separated from AGS by pathological findings and clinical features, and could be diagnosed as EHBA and not as atypical AGS. The increase of interleukin 8 (IL-8) production induced by tumor necrosis factor α (TNF-α) in Huh 7 cells was suppressed by the coexistence of JAG1 protein. We examined the different influences between wild-type cells and the 3 kinds of mutants detected in EHBA on Huh 7 cells and found that 2 of 3 mutants showed about half of the repressed activity compared with that of wild type. In conclusion, these results suggest that the JAG1 gene abnormality may be an aggravating factor in EHBA. (HEPATOLOGY **2002;36:904-912.**)

Alagille's Syndrome (AGS; Mendelian Inheritance in Man [MIM] 118450) was first reported as a paucity of intralobular bile ducts.[1] It is also known as arteriohepatic dysplasia. Intrahepatic bile duct atresia and pulmonary stenosis are critical findings in addition to cholestatic symptoms other than the remaining symptoms, vertebral abnormalities, embryotoxin, characteristic facies, and renal involvement. Mutations in the human jagged 1 (JAG1) gene have been reported to be responsible for AGS,[2,3] with an autosomal-dominant inheritance and with the ratio of the gene abnormalities reaching 60% to 75%.[4-11] Whether the JAG1 mutation in AGS is related to hepatic symptoms or cardiac malformation is an important question.

JAG1-Notch interaction has been reported as important for cell-to-cell regulation and also for cell differentiation of hematopoetic[12] and immunologic puripotent cells.[13] In experiments of jagged 1 knockout mice, the homozygous mice die from hemorrhage during early embryogenesis, and heterozygous mice with the jagged 1 null allele exhibit an eye dysmorphology.[14] Moreover, 2 recent studies reported that JAG1 abnormalities are detected in a few subclinical AGS families who suffer mainly from cardiac malformation in 2 generations.[15,16] The studies also reported that the expression patterns of JAG1 on the cardiac pulmonary region during the embryonal stage are correlated with congenital cardiovascular defects observed in AGS.[17-19] These data suggest the JAG1 mutation is mainly related to cardiac malformation.

Both extrahepatic biliary atresia (EHBA) and AGS, however, are recognized as developmental abnormalities

Abbreviations: AGS, Alagille syndrome; JAG1, human jagged 1; EHBA, extrahepatic biliary atresia; SSCP, single strand conformation polymorphism; cDNA, complementary DNA; TNF-α, tumor necrosis factor α; IL-8, interleukin 8; NF-κB, nuclear factor κ B.

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of the bile duct. As for the etiology of EHBA, 2 main hypotheses have been proposed: early embryonic malformation and perinatal invasion of a presumed inflammatory process.[20] Virologically, several causative agents have been proposed, including cytomegalovirus[21] or reovirus type 3.[22] Several studies have examined the genetic predisposition of EHBA in twins,[23] siblings,[24] and 2 generations of one family.[25] However, the majority of EHBA infants are sporadic cases, and analysis of twins showed that the coincident provocation of EHBA is limited to 1 of 8.[26] These findings could not support the mutation of a gene as the cause of EHBA. The proposed mechanism of bile duct development is a process of epithelial deletion and mesenchymal proliferation that is essential for the formation of normal bile ducts.[27,28] Complexity suggests that an inherent or acquired imbalance element leads to abnormal biliary structures in EHBA. The JAG1-Notch pathway affects not only the regulation of cell differentiation but also organogenesis through the expression of lineage-specific genes on the cells.[28,29] The detection of the messenger RNA of JAG1 around the disk plate during the embryonal stage[30] suggests the possibility that the JAG1 gene affects bile duct construction (*i.e.*, the disease process of EHBA). A similar phenotype of EHBA to AGS and the role of JAG1 on the bile duct formation process supports the examination of the JAG1 mutation in EHBA. This study aims to clarify the roles of the JAG1 gene on the pathogenesis of EHBA and to suggest a mechanism of an abnormal JAG1 protein in the appearance of the inflammatory process.

Patients and Methods

Subjects. The 102 EHBA subjects included 28 patients treated with transplantation at less than 5 years of age and 74 patients treated only with a Kasai surgery and good prognosis. All patients were diagnosed by using the pathologic findings of liver and extrahepatic bile ducts at the time of the Kasai surgery. To exclude the misdiagnosis of EHBA patients with the JAG1 mutation as AGS, we carefully examined the clinical findings, including the cardiac, vertebral, and ocular examinations by each specialist. In addition, their family histories and medical records spanning more than 5 years were reviewed. We excluded those patients who died within less than 5 years because of possible confusion with AGS patients. For the genetic study, we consulted with the patients and their parents, and obtained written informed consent, approved by our hospital committee.

Mutation Analysis. Sequence analysis was performed with cloned DNA fragments from 124 blood or 32 biopsy specimens from the patients. For single strand conformation polymorphism (SSCP) analysis, we used 100 healthy volunteer controls concomitantly. The sequences were determined with the procedures of SSCP and the direct and/or cloning sequencing methods reported previously.[4] A complete coding region of the JAG1 was amplified by a set of 31 primer pairs with a radioactive procedure using [32P] deoxyadenosine triphosphate (Amersham, Buckinghamshire, UK) in an automatic polymerase chain reaction programmer (Astec Co. Ltd., Fukuoka, Japan). The SSCP results were analyzed by Fujix Bastation version 1.2 in an image analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan). Cycle sequencing of the purified products was performed with the forward and reverse primers of the corresponding exon with the M4 primer and Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science, Inc.) according to the manufacturer's instructions. The JAG1 complementary DNA (cDNA) and protein sequences were taken from DDBJ/EMBL/GeneBank (accession no. AF003837). Mutation analysis of JAG1 was performed using Genetyx-Mac version 8.0 software (Genetyx Co. Ltd., Tokyo, Japan).

DNA Constructs of JAG1 Mutants. The cDNA clone of full-length JAG1 was ligated at the Sph I, Sac II, and Bgl II points by the restriction enzymes technique in the pUC[18] vector. This cDNA was sequenced by an Astec automatic PCR programmer (Astec Co.) with some modifications and was confirmed to show no mutations. Three kinds of mutants of JAG1 cDNAs were constructed by recombination of the cDNA of the wild-type JAG1 gene. The mutants of JAG1-flag tag constructs were constructed by using a QuickChange site directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) by using a PfuTurbo DNA polymerase and temperature cycler. Three kinds of oligonucleotide primers, each complementary to opposite strands of the vector, were extended by the cycler for 20 minutes at 16 cycles and digested by Dpn I endonuclease to remove the parent vector. The mutations containing a nicked vector were transformed into XL1-Blue supercomponent cells. The presence of the mutations was checked by sequencing several clones of the mutants. The isolated encoding region fragments from their cDNAs at the BssHII and XhoI points were subcloned in an expression vector. Their orientation and code arrangements were confirmed by DNA sequencing.

Stable JAG1 and Its Mutant Gene Transfection. Transfection was performed with SuperFect Transfection Reagent (Qiagen Inc., Hilden, Germany) following the manufacturer's protocol. Wild-type JAG1 and its mutations with FLAG were transected into 3T3 cells with the p3XFLAG-CMV-10 expression vector (Sigma Co., St. Louis, MO). Five micrograms of purified plasmid DNA of each JAG1 gene was used for transfection of 5×10^5

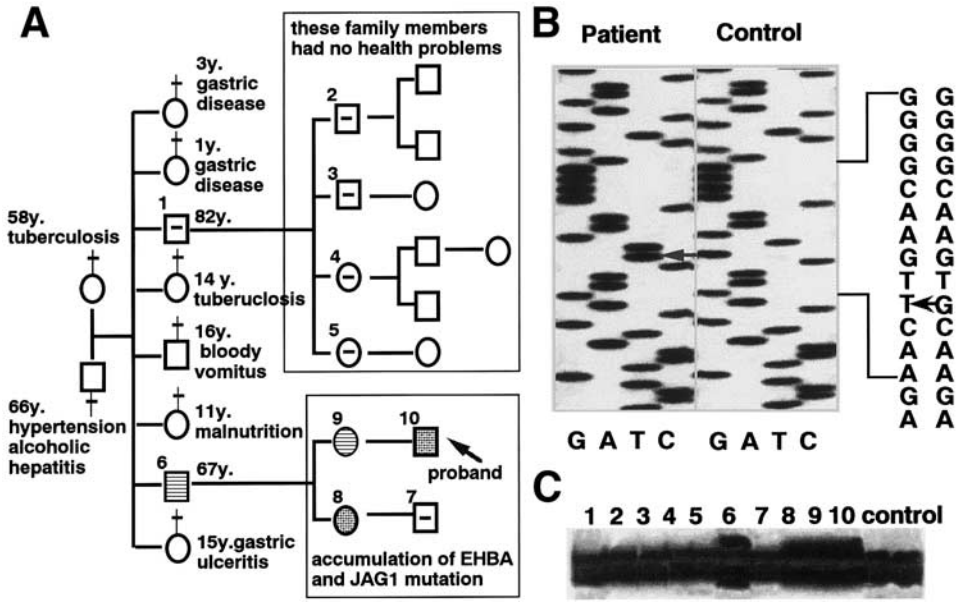

Fig. 1. Pedigree of a family consisting of a proband and his aunt who suffered from EHBA. (A) Hereditary correlation between EHBA and JAG1 mutation. (▭), JAG1 gene mutation not detected; (▤), JAG1 gene mutation detected; (▦), EHBA patients with the JAG1 missense mutation; ○ and □, not examined. The numeral and letters on each case correspond with the result of SSCP analysis. Note that the grandfather's (no. 6) siblings died at less than 20 years of age except for one elder brother (no. 1) who showed no abnormalities in the JAG1 gene and whose offspring (**upper square enclosure**) were all healthy. In the **lower square enclosure**, we confirmed the JAG1 mutations of 4 members (nos. 6, 8, 9, and 10) and 2 offspring (nos. 8 and 10) who suffered from EHBA. (B) Mutational analysis in the proband with the JAG1 mutation of those who suffered from EHBA. Missense mutation on exon 2, 592T→G was detected by DNA sequencing of the cloned DNA fragments. (C) SSCP analysis. The analysis of this family member revealed an abnormal band found in the grandfather (no. 6), mother (no. 9), aunt (no. 9), and proband (no. 10).

cells seeded in a 60-mm dish. The cells were cultured for 48 hours at 37°C and 5% CO_2, and transferred to two 100-mm dishes containing selective medium with 1.5 mg/mL neomycin (Meiji Co. Ltd., Tokyo, Japan). Positive clones were selected after an additional 4 to 8 weeks of culture. We confirmed stable JAG1-transfected cells by the polymerase chain reaction method at the genomic DNA level with a pair of primers that skipped one intron of the JAG1 gene and by Western blotting analysis of the FLAG protein. Each stable cell line was selected and applied to analyze the effect of missense mutations.

The Suppressive Effect of JAG1 and Its Mutants on the Interleukin 8 Production. Cell lines of Huh 7 and 3T3 were provided by the Tohoku Cell Culture Center, Sendai, Japan. 3T3 cells (5×10^5) transfected with 3 kinds of mutants and wild-type JAG1 were cultured in RPMI with 10% fetal calf serum for 48 hours. The 3T3 cells and the supernatant were collected separately. Each supernatant (100 μL) was incubated overnight in a well coated with anti-FLAG antibody. After blocking the plate with 3% bovine serum albumin, the medium was changed to RPMI with 10% fetal calf serum, and the Huh 7 cell (5×10^5/well) was cultured with or without the tumor necrosis factor α (TNF-α) addition of 500 pg/mLμ for 24 and 48 hours. As another method, THP-1 cells were cocultured with 3T3 cells transfected by wild-type or by 3 kinds of mutants. Instead of the supernatant, each separated 3T3 cell (2.5×10^5/well) was cocultured with Huh 7 cells (5×10^5/well) under the same condition as the supernatant applied method. The concentration of interleukin 8 (IL-8) in each supernatant was measured by OPT EIA Human IL-8 SET (Pharmingen, San Diego, CA).

Results

Detection of JAG1 Gene Abnormalities in Families With Plural EHBA Patients. Our hospital followed-up 3 families with several members afflicted with diseases of the extrahepatic bile duct. Two families with 2 siblings suffered from EHBA and extrahepatic choledochal cysts, respectively, and one family that involved 2 EHBA patients; the proband and his aunt. In the former 2 families, we could not detect any abnormality in the JAG1 gene. In the latter family, a mutation in the JAG1 gene was detected that originated from their grandfather (Fig. 1). The same mutations located at the DSL region (exon 2, 592G→T) were found in the proband, mother, aunt, and the grandfather; and 2 members, the aunt and proband, suffered from EHBA. The aunt underwent liver transplantation at childbirth, and her offspring showed normal liver function without the abnormality of the JAG1 gene.

Table 1. Mutational Analysis in 9 Cases of EHBA With the JAG1 Mutation

Gender Age	Transmission	Transplanted Age	Prognosis	Mutation Type	Location	Nucleotide Change	Affected Locus	Aminoacid Change
Case 1								
M, 12 y	Familial	Not yet 10 y	Poor	Missense	Exon 2	592 G → T	5′ DSL	V45L
Case 2								
F, 22 y	Familial	Transplanted at 22 y	Transplantation	Missense	Exon 2	592 G → T	5′ DSL	V45L
Case 3								
M, 6 y	Sporadic	No problem	Good	Missense	Exon 2	616 A → G	5′ DSL	N53D
Case 4								
M, 7 y	Sporadic	Transplanted at 2 y 7 mo	Transplantation	Missense	Exon 2	653 A → T	5′ DSL	K65M
Case 5								
F, 6 y	Sporadic	Transplanted at 1 y 9 mo	Transplantation	Missense	Exon 4	1067 G → A	DSL	R203K
Case 6								
F, 5 y	Sporadic	Transplanted at 10 mo	Transplantation	Missense	Exon 16	2527 T → G	EGF	Y690D
Case 7								
F, 5 y	Sporadic	Transplanted at 1 y 3 mo	Transplantation	Missense	Exon 22	3183 C → G	CR	H908Q
Case 8								
M, 6 y	Sporadic	Transplanted at 2 y 1 mo	Transplantation	Missense	Exon 22	3221 T → C	CR	L921P
Case 9								
M, 6 y	Sporadic	Transplanted at 2 y 6 mo	Transplantation	Missense	Exon 26	4097 G → A	IC	R1213Q
Case 10								
F, 5 y	Sporadic	Transplanted at 1 y 3 mo	Transplantation	Missense	Exon 22	3071 G → C	CR	P871R
Case 11								
M, 6 y	Sporadic	Transplanted at 2 y 1 mo	Transplantation	Missense	Exon 22	3071 G → C	CR	P871R

Abbreviations: 5 DSL, from Signal peptide to DSL region; IC, intracellular region.

The clinical course of the proband was stable with recognized hepatic cirrhosis, portal hypertension, and slight jaundice. The grandfather was diagnosed with mild alcohol-induced hepatitis, and the mother of the proband showed normal liver function. Ten offspring of the living mother's uncle without the JAG1 mutation did not show any hepatic dysfunction. The correspondence ratio of JAG1 gene abnormalities with EHBA was less than 50%. This ratio does not suggest that the JAG1 gene is responsible for the EHBA, but rather suggests a relation with the exacerbation or development of the disease.

Examination of Abnormalities of the JAG1 Gene in Sporadic EHBA Cases. Next, we performed a gene analysis of 98 sporadic EHBA patients. The patients were divided into 2 groups consisting of those with or without liver transplantation at less than 5 years of age. The positive mutation ratio in patients without transplantation was 1 of 70. On the other hand, the ratio of transplanted patients was 6 of 28. The mutations were all missense mutations with one amino acid replacement (Table 1). The mutations found in EHBA patients were not detected in 100 control samples. Otherwise, the mutation site exon 22, 3071G→C, was also detected in 2 nonrelated EHBA patients (Table 2). We classified this mutation as a polymorphism that was reported previously[10,31] that showed the same effect on the function of wild type in cell differentiation experiments.[32]

Differential Diagnosis of EHBA From AGS. The clinical and laboratory findings of the 9 EHBA patients with missense mutations revealed a progressively worse clinical course with continuous increases in direct bilirubin and the presence of acholic stool around 3 months of age. None of the patients exhibited major symptoms of AGS even after 5 years of age. All patients underwent the Kasai surgery at least once. During their surgery, the occlusion or absence of a biliary tract was confirmed by macroscopic observation (Table 3). These clinical and extrahepatic findings were not compatible with AGS.

In addition, their histologic findings by liver biopsy during the Kasai surgery were distinguishable from those of AGS. The hyperplasia of intrahepatic bile duct was marked in all samples of the 9 cases. The mean ratio of the

Table 2. Polymorphisms Identified in the JAG1 Coding Sequence

Exon	Nucleotide Polymorphism	Amino Acid	Appearance Rate %
2	726 G → A*	G89	1
4	1032 C → T	Y191	1
4	1047 C → T*	C196	1
4	1139 G → C	C227P	1
5	1203 A → G*†	P248	1
6	1224 C → T*†	Y255	2
17	2589 T → C	D710	2
17	2673 A → C*†	T738	1
22	3071 G → C*†	P871R	2
22	3137 C → G	L904	1
26	3987 T → C*	Y1176	1

*Polymorphisms identical to those summarized by Spinner et al.[31]
†Polymorphisms identical to those reported by Heritage et al.[9]

Table 3. Clinical Symptoms and Laboratory Findings in EHBA Cases With JAG1 Missense Mutation

Case No. (Gender, Age)	Initial Symptom: Alcoholic Stool/Jaundice	Laboratory Test: Gallbladder by Echogram/Finding of Duodenal Fluid	Macroscopic Findings: Findings at Operation	Laboratory Findings at the Kasai Operation: AST/ALT (IU/L)	γ-GTP (IU/L)/ T-chol (mg/dL)	T-Bil/D-Bil (mg/dL)	Malformations: Major Five Symptoms of AGS and Other Abnormalities
Case 1 (M, 12 y)	Present/present	Absent/transparency	Absent gallbladder, fibrous biliary tract	125/88	232/221	12.6/9.8	face(−), bone(−), PS(−), eye(−)
Case 2 (F, 22 y)	Present/present	ND/transparency	Atretic gallbladder, patent biliary tract	202/167	156/201	9.8/8.1	face(−), bone(−), PS(−), eye(−)
Case 3 (M, 6 y)	Absent/present	Absent/transparency	Absent gallbladder, fibrous biliary tree remnant	46/37	369/211	8.7/7.0	face(−), bone(−), PS(−), eye(−)
Case 4 (M, 7 y)	Present/present	Present/transparency	Operative cholangiogram; biliary tract atresia; white bile	268/186	ND/183	7.4/6.0	face(−), bone(−), PS(−), eye(−)
Case 5 (F, 6 y)	Present/present	Absent/transparency	Absent gallbladder, fibrous biliary tree remnant	91/59	285/145	8.2/6.3	face(−), bone(−), PS(−) (Heart murmur detected at one month old), eye(−)
Case 6 (F, 5 y)	Present/present	Absent/transparency	Absent gallbladder, fibrous biliary tree	131/101	170/174	9.1/4.5	face(−), bone(−), PS(−), eye(−)
Case 7 (F, 5 y)	Present/present	Absent/transparency	Absent gallbladder, fibrous biliary tree	125/88	156/198	8.1/6.1	face(−), bone(−), PS(−), eye(−)
Case 8 (M, 7 y)	Present/present	Absent/transparency	Absent gallbladder, striae structure of the biliary tract	89/96	124/201	6.9/4.2	face(−), bone(−), PS(−), eye(−)
Case 9 (M, 6 y)	Present/present	Absent/transparency	Absent gallbladder, fibrous biliary tract	66/96	276/201	11.1/9.6	face(−), bone(−), PS(−), eye(−)
Case 10 (F, 5 y)	Present/present	Absent/transparency	Absent gallbladder, striae structure of the biliary tract	222/145	271/204	9.8/7.8	face(−), bone(−), PS(−), eye(−)
Case 11 (M, 6 y)	Present/present	Absent/transparency	Absent gallbladder, fibrous biliary tree remnant	129/102	221/199	9.1/6.8	face(−), bone(−), PS(−), eye(−)

Malformations: face, characteristic face; bone, spina bifida; PS, peripheral pulmonary stenosis; eye, posterior embryotoxon.
Abbreviation: ND, not determined.

number of bile ducts for a portal area was 5.8 ± 2.3 (range, 4.1-9.2) (Fig. 2A). The extrahepatic bile duct was obstructed by granulation and fibrosis, and many inflammatory cells and increased bile ductules were observed around it (Fig. 2B).

Genetic Characteristics of EHBA With JAG1 Mutations. As reported by Spinner et al.[31] and including our data,[33] in AGS, a total of 243 mutations of AGS have been reported. Fig. 3 shows the ratio of the mutation divided by the 6 domains of JAG1, composed of 33 missense and 210 other than missense mutations. The loci of the mutations in the 5 EHBA cases that included 2 consanguineous ones were deviated to the 5′ DSL-DSL region. This finding corresponds to the distribution of the AGS missense mutation, in which the 5′ DSL is the most frequent mutation locus.

The ratio of the mutation in EHBA was found to be approximately 9% and was different by prognosis, 6 of 28 in those transplanted at under 5 years old and 3 of 72 in the follow-up group without transplantation, respectively. Consanguinity analysis revealed that the ratio of detection of the JAG1 mutation in the former group was significantly higher than that of the latter group ($P < .05$, $x^2 = 5.985$). The latter group included the 2 familial EHBA patients. One of them underwent transplantation at the age of 20 years and the other's prognosis was estimated to be poor. These findings suggest that the role of the JAG1 mutation in EHBA could work as an aggravation factor.

Partial Defect of Mutants in JAG1 Activity on Cytokine Regulation. Morrissette et al.[32] examined the mutants detected in AGS and found that 2 of 4 kinds of mutants revealed the partial dysfunction of the JAG1 protein. We also checked the 3 kinds of mutants found in EHBA, 3071G→C on the CR region that was thought to be a polymorphism, 592G→T on the DSL region, and 3221T→C on the CR region. We found that TNF-α induced the IL-8 production of Huh 7 cells, and that this effect was suppressed by the existence of the JAG1 protein. The activity of JAG1 mutants was measured by 2 methods; the coculture with 3T3 cells transfected with wild type and mutants of the JAG1 gene, and the addition of supernatant from each cell culture. The effects using both methods revealed the same tendency but the sup-

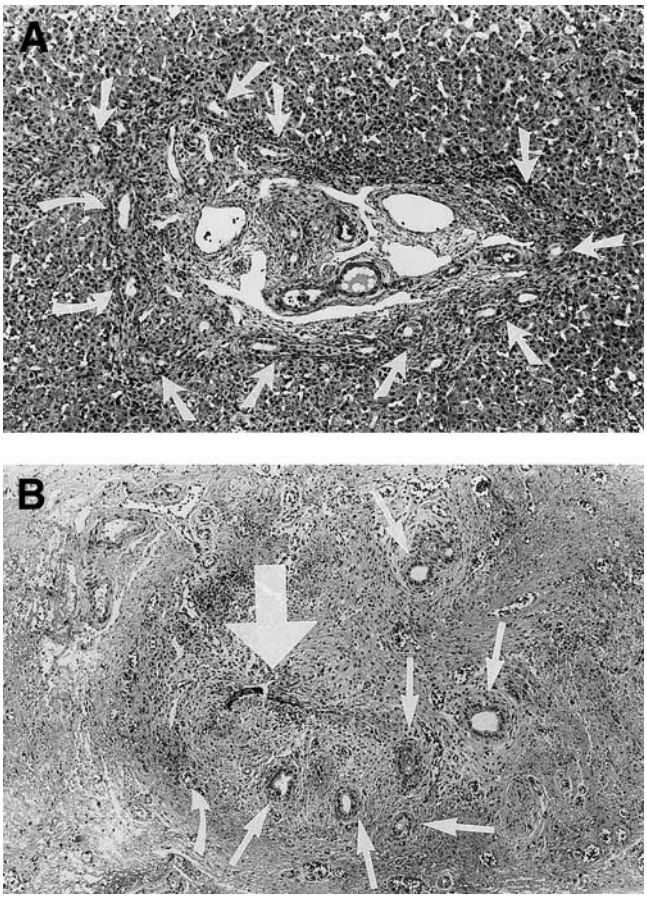

Fig. 2. Pathologic findings of the intrahepatic and extrahepatic bile duct of EHBA patients with the JAG1 mutation. (A) Pathologic findings of the liver. The hyperplasia of the intralobular bile ducts is evident in the portal area, and inflammatory cells circumferentially infiltrate around the bile ducts (**arrow**). Original magnification ×200. (B) Pathologic findings of remnants of extrahepatic bile ducts. The main extrahepatic bile duct (**large arrow**) is destroyed and obliterated by inflammatory granulation tissue and fibrosis leaving only tiny glands (**small arrow**) around it. Original magnification ×200.

pression effect of the coculture method was higher than that of the addition of the supernatant. Two mutants (mutants 592 and 3221) showed half the activity compared with that of wild type. Mutant 3071 repressed the IL-8 production similar to wild type (Figs. 4A and 4B).

Discussion

There are 2 essential points of the present study: one is the diagnostic possibility that EHBA with the JAG1 mutation is atypical AGS, and the other is the suggested mechanism by which the JAG1 mutation works as an aggravation factor in EHBA.

Concerning the differential diagnosis of EHBA and AGS, clinically the 9 EHBA cases with JAG1 mutation did not show any major symptoms of AGS after 5 years, and pathologically revealed different intra- and extrahepatic bile duct findings from those of AGS. Cardiac defects existed in 7% of EHBA,[20] and in the present 102 EHBA cases there were only 3 cases with cardiac involvement, which included a ventricular septal defect, an endocardial cushion defect, and arrhythmia, without any JAG1 gene mutations. Other various anomalies reportedly are associated with EHBA,[20] but no congenital anomalies were found in these 9 patients with the JAG1 mutation.

Pathologically, the hyperplasia of the intrahepatic bile duct was marked in all 9 cases. The increased number of intrahepatic bile ductules in the present EHBA cases might relate to poor prognosis because the duration of bile stasis is correlated with the number of ductules in EHBA.[34] In some AGS cases, it was reported that intrahepatic bile ducts do not disappear at early infancy,[35,36] but, even in these cases, the extrahepatic biliary tract was not obstructed. Thus, the pathologic findings of AGS

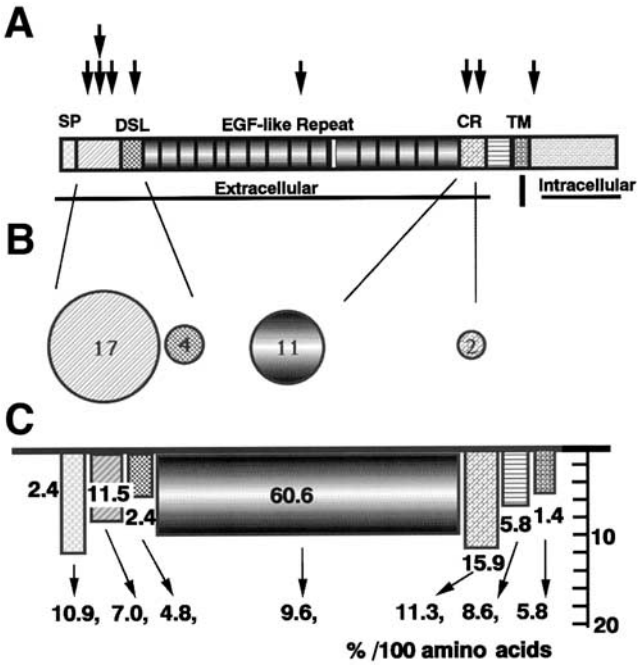

Fig. 3. Comparison of the mutation locus found in EHBA and AGS. JAG1 ligand consists of 6 conserved motifs composed of a signal peptide (SP) domain, a Delta/Serrate/Lag-2 (DSL) domain, 16 epidermal growth factor (EGF)-like repeats, a cysteine-rich (CR) region, a transmembrane (TM) domain, and an intramembranous and intracellular domain. The **upper arrows** show JAG1 mutations found in EHBA cases with JAG1 mutation and the location of the locus. The size of the circle shows the number of AGS cases with missense mutation located in each domain. The numerals in the circle indicate the number of cases with missense mutation. The frequency of the missense mutation was deviated to the 5′ region of DSL. The area of bars and the numerals enclosed reveal the frequency of the mutation in each domain, and their heights and arrowhead numerals indicate the calculated mutation ratio to 100 amino acids (numbers of reported mutations/numbers of amino acids contained in each locus × 100). The provocation ratio of mutations, excluding the missense mutations in AGS, occurred comparatively evenly.

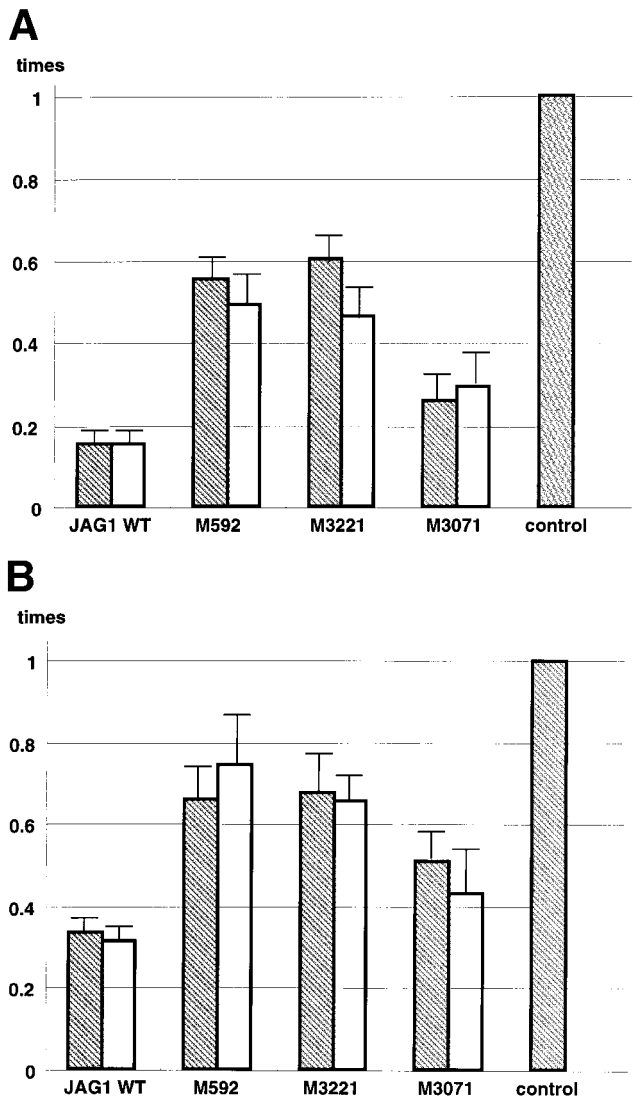

Fig. 4. Effect of JAG1 gene products on IL-8 production of the Huh 7 cell line. **Bars** show the mean values of IL-8 suppressive ratio by various kinds of 3T3 cells, in 24 hours (**slashed bar**) and 48 hours (**open bar**). The height of each bar represents the ratio compared with control and mean and standard deviation of triplicate determinations. Subtracting the IL-8 concentration without stimulation of TNF-α from that with stimulation was defined as the increase of IL-8. The suppressive ratio of the 3T3 cells on the increase of IL-8 was calculated by dividing it by that of the control. Control, 3T3 cells without transfection; JAG1WT, JAG1 wild type; M592, JAG1 mutant 592G→T; M3221, JAG1 mutant 3221T→C; M3071, JAG1 mutant 3071G→C. (A) Effect of the JAG1 mutants on IL-8 production by coculture method. IL-8 production of Huh 7 cells stimulated by TNF-α was measured under the condition of coculture with various 3T3 cells. 3T3 cells were transfected by the 3 kinds of mutants and wild type of JAG1. For controls, the Huh 7 cells were cocultured with the 3T3 cells without transfection. The 2 kinds of 3T3 cells transfected with mutant JAG1 gene (M592 and M3221) suppressed the increase of IL-8 production of Huh 7 cells by stimulation of TNF-α less effectively than that with wild type. (B) Effects of JAG1 protein on IL-8 production by the supernatant method. The supernatants of 3T3 cells transfected with the 3 kinds of JAG1 genes and wild type were applied to an anti-FLAG antibody coating plate. Huh 7 cells were cultured in the wells coated with 4 kinds of JAG1 proteins for 24 (**slashed bar**) and 48 hours (**open bar**). The suppressive activities of both M592 and M3221 were reduced to half compared with those of wild type and M3071.

differed from those of our cases with intrahepatic biliary hyperplasia and obstructed extrahepatic bile ducts.

The next problem is how to relate the JAG1 gene with the pathogenesis of EHBA. In our hospital, the success rate of Kasai's procedure is 72% with continued improvement of jaundice after the surgery. Even in unsuccessful cases, usually the symptoms of bile discharge (*e.g.*, decrease of jaundice and colored stool) develop soon after the surgery, but the improvement tendency dissipates because of reocclusion with hyperplasia of intestinal epithelial cells on the porta hepatis. Therefore, the main reason for necessitating transplantation at less than 5 years in EHBA patients is due to the postoperative reactive processes. The process of reocclusion is determined by the competition between the strength of cholic flow and granuloma formation at the porta hepatis. If JAG1 mutations could influence these parameters, it might help explain the relation between the reobstruction in EHBA and JAG1 abnormalities.

Two hypotheses could explain the deviated detection of JAG1 abnormalities in serious EHBA. The first is that the JAG1 gene influences duct formation during the embryonal stage, and that the mutations found in EHBA induce the malformation or dysfunction of the intrahepatic bile duct as a result. If the JAG1 gene product influences duct formation during the embryonal stage,[30] as reported in other organs,[37,38] the mutations found in EHBA should also induce dysfunction of the intrahepatic bile duct. Because missense mutations were also detected in a total of 33 AGS cases,[3-11,33] the missense abnormality of the JAG1 gene could be responsible for the malformation of the intrahepatic bile ducts. The intrahepatic bile flow of EHBA patients with the mutation might also be disturbed as was that of AGS, which brings about reocclusion and poor prognosis, resulting in the necessity for transplantation as the final therapy.

The second hypothesis is based on the assumption that the JAG1 gene involves the repression of inflammatory mechanisms in the liver. It was reported that nuclear factor κB (NF-κB) activation induces up-regulated expression of the JAG1 protein.[39] In addition, the activation of the Notch signal suppresses the NF-κB2 signaling transduction.[40] The relation between the JAG1-Notch signal and cytokine production is summarized as a negative feedback system.[41] The inflammatory cytokines work on increasing JAG1 protein on the cell surface through NF-κB activation, and, reversibly, the increased JAG1 expression stimulates the Notch signal pathway. These processes result in the suppression of NF-κB and in the reduction of the inflammatory cytokines.[41,42] We examined the effects of JAG1 protein on the cytokine production of the Huh 7 hepatoma cell line and found that this

protein suppressed the accelerated production of IL-8. Moreover, a partial loss of the effects was observed in 2 kinds of mutations found in EHBA. The mutants of the JAG1 gene produce abnormal proteins that affect inflammatory processes in the liver via the JAG1-Notch pathway regulation of the cytokine network. When these findings are applied to the role of the JAG1 gene on EHBA, it is possible that the poor prognoses of EHBA patients with JAG1 mutations might be explained by insufficient regulation of inflammatory cytokines caused by dysfunction of the JAG1-Notch pathway. Even if other factors, such as some viruses or organisms, are responsible for EHBA, the key to determine the prognosis is the continued inflammation of the liver. Consequently, because the continued activation of inflammatory cytokines is one of the risk factors, the abnormality of the JAG1 gene could aggravate the prognosis of EHBA.

The present findings detecting the missense mutation mainly in serious EHBA cases suggest such mutations work as an aggravation factor of EHBA. It is suggested that the JAG1 mutation not only determines the clinical course in EHBA, but also works as a risk factor for hepatitis.

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