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Genetic alterations in the *JAG1* gene in Japanese patients with Alagille syndrome

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Abstract Alagille syndrome (AGS) is a congenital anomaly syndrome that affects liver, heart, pulmonary artery, eyes, face, and skeleton. Recently, mutations of the *JAG1* gene, which encodes a ligand for the Notch receptor, have been identified in AGS patients. We investigated the *JAG1* gene for genetic alterations in eight Japanese AGS patients, using fluorescence in situ hybridization (FISH), single strand conformation polymorphism (SSCP) analysis, and direct sequencing. Subtle genetic alterations were identified in six of the eight patients, including three frameshift mutations, two splice donor mutations, and one nonsense mutation. All alleles with identified mutations can be expected to produce non-functional truncated proteins without a transmembrane domain. There was no apparent correlation between the genotypes of the patients and their affected organs, although the phenotypes of the patients with mutations at the splice donor site were found to be less severe.

Key words Alagille syndrome · *JAG1*

Introduction

Alagille syndrome (AGS; MIM No.118450) is characterized by chronic cholestasis associated with paucity of interlobular bile duct (PILBD), congenital cardiac defects, posterior embryotoxon, characteristic facial appearance, and butterfly vertebrae (Alagille et al. 1975). It is an autosomal dominant disorder with a penetrance estimated at 94% (Dhorne-Pollet et al. 1994). Following a study of linkage analysis in three generations of an AGS family (Hol et al. 1995), and of AGS patients with submicroscopic deletion or with balanced translocation (Deleuze et al. 1994; Rand et al. 1995; Pollet et al. 1995; Spinner et al. 1994), the chromosomal region responsible for AGS was localized at 20p12. Recently, the *JAG1* gene, which encodes a ligand for the Notch receptor and plays a role in determining cell fates in early embryonic development, was mapped to the AGS critical region, and its mutations in AGS patients were reported by two groups (Li et al. 1997; Oda et al. 1997). In this study, we investigated the *JAG1* gene for genetic alterations in eight Japanese patients with AGS.

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Patients and methods

Patient samples

Blood samples of eight AGS patients were obtained with informed consent in accordance with established standards. Patients with at least three of five major features of the syndrome were diagnosed as having AGS by a pediatric hepatologist and a dysmorphologist. The clinical characteristics of the patients are summarized in Table 1. Patient 1 had an affected sibling, while patient 2 had two siblings who had already died of AGS. Blood samples from unaffected family members of patients 1, 2, 3, 4, and 6 were also obtained. Genomic DNA was extracted from peripheral blood leukocytes under standard conditions.

Fluorescence in situ hybridization (FISH)

Metaphase chromosomes were prepared from a lymphoblastoid cell culture of the patients. FISH was carried out by the standard method. YAC clone 940d11, which

Table 1. Summary of clinical features and mutations

Patients	1	2	3	4	5	6	7	8
Major features								
Chronic cholestasis	+	+	+[a]	+	+[a]	+	+[b]	+
PILBD	+	+	+/−[c]	+/−[c]	+	nd[d]	+	+
Characteristic facial features	+	+	+	+	+	+	+	+
Cardiac abnormality	+	+	+	+	−	+	+	+
Vertebral abnormality (butterfly-shaped)	−	−	−	−	+	+	−	−
Posterior embryotoxon	−	−	−	−	−	−	−	−
Minor features								
Mental retardation	−	−	−	−	−	−	−	−
Growth retardation	+	+	−	−	+	+	−	+
Other skeletal abnormalities (vertebral)	−	−	−	−	−	−	−	+
Renal involvement	−	−	−	−	−	−	−	−
Family history	+[e]	+[f]	−	−	−	−	−	−
Mutation	ND[g]	2094delA	ND[g]	2103delC	694 + 2T → A	2122–2125del	755G → A	R774X

PILBD, Pancity of interlobular bile duct
[a] + Patients were affected with hyperbilirubinemia in their neonatal period
[b] + The cholestasis in the patient has regressed spontaneously
[c] +/− Apparent PILBD has not been observed in the patient's liver biopsy specimen
[d] nd, Liver biopsy was not done because of refusal
[e] + The patient had an affected sibling
[f] + The patient had two siblings who had already died from AGS
[g] ND, Not detected

includes the entire genomic region of the *JAG1* gene, was used as a probe (CEPH Mega YAC library). Total yeast DNA was labelled with biotin-16-dUTP by nick translation and was detected with avidin-fluorescein isothiocyanate (FITC) (Boehringer Mannheim, Berlin, Germany). The chromosomes were then counterstained with propidium iodide (PI) (Sigma, St Louis, MO, USA) in an anti-fade solution.

Single strand conformation polymorphism (SSCP)

Polymerase chain reaction (PCR)s of 26 exons of *JAG1* were performed, using 31 sets of previously described primers (Oda et al. 1997). Electrophoresis was carried out in acrylamide gels containing 0.5 × Tris-borate/EDTA electrophoresis buffer (TBE), at 500 W in a room maintained at 4°C. The gels were stained with SYBR GreenII (FMC Bio Products, Rockland, ME, USA) for 10 min, and then bands were visualized and analyzed with an FMBIOII Multi-View fluorescent image analyzer (Takara, Tokyo, Japan).

Direct sequence analysis

Direct DNA sequencing of PCR products from genomic DNA was carried out on both strands by the dideoxy chain-termination method with an ABI PRISM DNA sequencing kit (Applied Biosystems, Foster City, CA, USA). Reactions were analyzed with an ABI373S autosequencer according to the manufacturer's protocol (Perkin Elmer-Cetus, Norwalk, CT, USA).

PCR using mismatch primer and restriction enzyme digestion

A 1-bp deletion at nucleotide 2094 in the *JAG1* gene of patient 2 was screened in his family by digestion of the PCR product by *Mbo*II. We designed a set of primers that introduced an *Mbo*II site into the mutated sequence: E16muS (5′-TTAATGA CTGCAGCCAGAACCC-3′) for forward, and E16muA (5′-CGTGAGTGGCAGGTCT TTTCTT-3′) for reverse. PCR conditions were 40 cycles of 30 s each at 94°C, 64°C, and 72°C. PCR products were digested with *Mbo*II according to the manufacturer's (Takara, Tokyo, Japan) protocol. Migration was performed on a 12% polyacrylamide gel at 150 V for 2 h.

Results

FISH analysis probed with YAC 940d11 revealed that none of the eight patients had chromosomal deletion at the AGS locus. This was followed by SSCP analysis for each of the 26 exons of *JAG1*. The patients whose samples represented aberrant bands on SSCP analysis were analyzed further by direct DNA sequencing of the PCR product from the genomic DNA.

Sequence analyses of exon 16 demonstrated a 1-bp deletion in patients 2 and 4, at nucleotide 2094 (2094delA) in the former and at nucleotide 2103 (2103delC) in the latter, while a 4-bp deletion in exon 17 (2122–2125del) was identified in patient 6 (Fig. 1a,b, and d). These frameshift mutations were expected to cause premature termination.

On the other hand, a T-to-A change in the second nucleotide of intron 4 (AACAG AG/gtatgtg to AACAGAG/gaatgtg) was observed in patient 5. This mutation causes the conserved splicing donor to change from GT to GA and can therefore be expected to cause abnormal splicing. In patient 7, a G-to-A change in the final nucleotide of

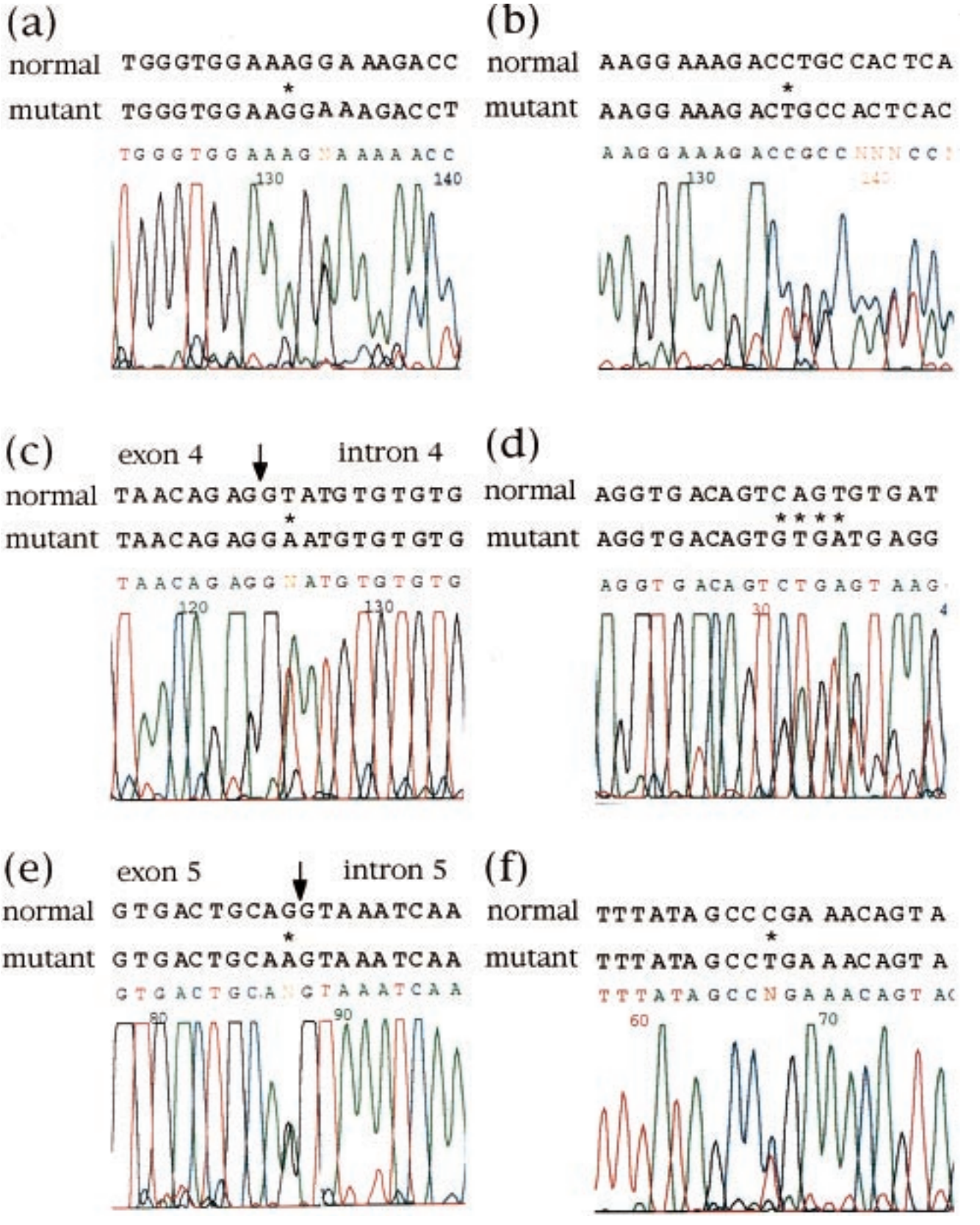

Fig. 1. a–f Sequence analysis of polymerase chain reaction (PCR) products from genomic DNA. *Asterisks* indicate deleted or substituted nucleotides and *arrows* indicate junctions of exon and introns. **a** Patient 2. In exon 16, a 1-bp deletion of "A" at nucleotide 2094 was identified. **b** Patient 4. In exon 16, a 1-bp deletion of "C" at nucleotide 2103 was identified. **c** Patient 5. A T-to-A change was observed in intron 4. **d** Patient 6. A 4-bp deletion of "CAGT" at nucleotide 2122 was identified in exon 17. **e** Patient 7. A G-to-A change at nucleotide 755 was identified in exon 5. **f** Patient 8. A C-to-T change at nucleotide 2230 was identified in exon 18. This substitution causes codon 744 to change from CGA(arginine) to termination codon TGA

exon 5 (ACTGCAG/gtaaatc to ACTGCAA/gtaaatc) was observed. Although this substitution alters the translation of codon 252 from AGG (arginine) to AAG (lysine), the sequence alteration immediately upstream of an exon/intron junction may interfere with the normal splice donor function. This sequence variation was not observed in any of 50 unrelated normal subjects (data not shown). These splice donor mutations were also expected to cause premature termination.

Finally, a C-to-T change at nucleotide 2230 in exon 18 was observed in patient 8. This substitution causes codon 744 to change from CGA (arginine) to a termination codon TGA. In patients 1 and 3, who did not show band aberration on SSCP analysis, direct sequencing of the entire coding region of *JAG1* was performed, but no mutation was identified.

Mutational analysis was also performed for the unaffected parents or siblings of patients 2, 4, and 6. To facilitate a screening for the identified mutation in patient 2, we designed a set of primers that introduced an *Mbo*II site into the mutated allele. Study of the patient's parents and siblings revealed that his mother, who is apparently unaffected, had the same mutation, while the others did not (Fig. 2b). Since sequence analyses of the parents of patients 4 and 6 revealed that they did not have the same mutations as the patients, it was concluded that the mutations in patients 4 and 6 were de novo.

The clinical features and the results of the mutational analyses in the eight patients are summarized in Table 1. All six mutations identified in this study were different from each other, and there was no apparent correlation between the type of the mutations and the affected organs. However, the phenotypes of patients 5 and 7, who both showed mutations at the splice donor sites, were less severe than those of the other patients.

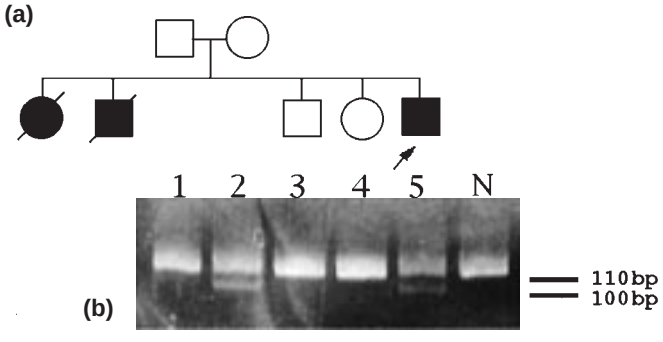

Fig. 2. **a** Pedigree of family of patient 2. *Squares* represent males, and *circles*, females. *Solid symbols* represent individuals with Alagille syndrome. *Diagonal slash* indicates that the individual is dead. *Arrow* indicates proband. **b** Screening of the 2094delA mutation in the family members of patient 2. *Lanes 1–4*, unaffected parents and siblings; *lane 5*, the patient; and *lane N*, normal control. The length of the PCR products was 110 bp, and mutant PCR products were expected to be digested with *Mbo*II into two fragments, 100 bp and 10 bp. In *lanes 2 and 5*, two types of fragment, with lengths of 110 bp and 100 bp, were observed, while only a 110-bp fragment was observed in the other lanes. The 10-bp fragments could not be identified under these conditions. The two fragments show different intensities because products with a heteroduplex cannot be digested with the enzyme

Discussion

We studied a total of eight unrelated Japanese AGS patients and found mutations in six of them. The mutations were identified as three frameshift mutations, two splice donor mutations, and one nonsense mutation, all of which can be expected to produce truncated proteins. The structural motif of *JAG1* has been previously described (Li et al. 1997; Oda et al. 1997), and the transmembrane domain of the *JAG1* protein is located near the C-terminal region. Thus, all putative truncated proteins should lack the transmembrane domain, and should lose their entire function as a ligand for the Notch receptor. Although we could not identify any chromosomal deletion by FISH analysis, AGS patients with large deletions, including the entire *JAG1* genomic region, have been reported (Anad et al. 1990). Recently, mutational analysis of *JAG1* in Japanese Alagille syndrome patients was reported (Yuan et al. 1998). Together with the data in that report, our data suggest that *JAG1* is also the gene responsible for AGS in the Japanese population.

The 1-bp deletions in patients 2 (2094delA) and 4 (2103delC) were located at a simple nucleotide repeat region, and the 4-bp deletion in patient 6 at a region where a pair of the four nucleotides "CAGT" lies in tandem. All of these deletions may have occurred during DNA replication as a result of slipping of the template strand and subsequent misalignment. A point mutation in patient 5 (694 + 2T->A) was identified at 2-bp downstream of the junction of exon 4 and intron 4. As GT represents a conserved sequence of the splice donor, this substitution may cause abnormal splicing. On the other hand, a point mutation in patient 7 (755G->A) was seen at 1-bp upstream of the junction of exon 5 and intron 5. This could be a simple missense mutation or a polymorphism that changes the translation of codon 252 from AGG (arginine) to AAG (lysine). Two cases of missense mutations of *JAG1* in AGS have been reported previously, and both cases had point mutations in codon 184 (arginine) which shows interspecies conservation (Krantz et al. 1998). The arginine residue of codon 252 is also conserved in rat *JAG1* and in human *JAG2*; however, because the sequence of AG/gta represents the consensus sequence of the donor splicing site, a sequence alteration immediately upstream of an exon/intron junction may constitute an obstacle to normal splicing. Because we could not obtain an RNA sample from this patient or DNA samples from his parents, we could not verify this hypothesis. However, since this substitution was not observed in 50 unrelated normal subjects, we considered that it induces abnormal splicing rather than that it constitutes a simple missense mutation or polymorphism. A similar splice mutation in the *JAG1* gene in an AGS patient was also reported by another investigator (Oda et al. 1997). There are some reported cases of abnormal splicing at donor splice sites with a 1-bp substitution at the −1 position by the use of cryptic splice site or by exon skipping (Krawczak et al. 1992).

In patients 1 and 3, we failed to find any mutations in the *JAG1* gene, even when the entire coding region was se-

quenced. Since we investigated only the coding region of the *JAG1* gene in this study, the possibility remains that the mutations in these patients lie within a non-coding region, e.g., the introns or promoter. Alternatively, genetic heterogeneity may be present in this disease, so that the diseases of the patients may have developed because of a mutation of the Notch receptor gene or another gene involved in Notch signal transduction.

The unaffected parents of three families were studied, and the unaffected mother of patient 2 was found to have the same mutation as that in the patient. Although we could not identify any mutation in the family members of patient 1, in which family both siblings have AGS, one of the parents should be also an asymptomatic carrier of AGS. Reportedly, the penetrance of AGS is 94% and 15% of the cases are sporadic (Dhorne-Pollet et al. 1994). Even though not so many cases were included in the present study, it can be said that previous estimates of the penetrance and the sporadic/inherited case ratio in this disease may be too high. Further studies of apparently unaffected family members of AGS patients should make this clear. To establish definitive ratios is crucial, because, when a carrier is detected in a given family, genetic counseling will become a clinically important issue.

We investigated correlations between the type of mutations and the patients' phenotype. The 4-bp deletion (2122–2125del) observed in patient 6 was the same mutation as that reported by Li et al. in 1997. However, the patient in that study had posterior embryotoxon, while our patient 6 did not have any ocular abnormality. The fact that different phenotypes are observed among patients with the same mutation, combined with the finding of an unaffected carrier of mutated *JAG1*, suggests that there is no apparent correlation between genotype and affected organs in AGS and that there may thus be other factors; (for example, other genes or environmental factors) which determine the phenotype of AGS patients. These results also support the finding that haploinsufficiency of the *JAG1* is responsible for the AGS phenotype, as suggested by other investigators (Krantz et al. 1998). However, the phenotypes of patients 5 and 7, whose mutations are located at the splice donor site, were found to be less severe than those of the other patients. As for the other genes, an allele with a mutation at the first nucleotide of the splice donor site produced no functional product, while mutations at other nucleotides of the donor site were seen to allow for the production of small amounts of normal protein (Gelehrter and Collins 1990; Krawczak et al. 1992). It is thus possible that the mutant alleles in patients 5 and 7 continued to produce small amounts of functional *JAG1* protein, resulting in their mild phenotypes.

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