rs17024525, 3980A>G, 4014C>T (McDaniell *et al.* 2006) as well as newly identified SNPs including 3779G>A and IVS8-6 G>A. A population survey of 100 ethnically matched controls revealed that four individuals were heterozygous for the 3779G>A substitution. The IVS8-6 G>A substitution was not found in 100 ethnically matched controls but the father of the Alagille syndrome patient with the IVS8-6 G>A substitution showed no signs of Alagille syndrome and found to have the substitution. Hence, the IVS8-6 G>A substitution is most likely a rare variant that does not cause disease. However, given the reduced penetrance of Alagille syndrome, it is possible that the IVS8-6 G>A substitution is disease causing yet the father was unaffected phenotypically.

Site-directed mutagenesis of known NOTCH2 *mutant alleles and their analysis by DHPLC*

PCR amplicons flanking the previously described mutant alleles of the *NOTCH2* gene were generated using the SOE-PCR method, and direct sequencing of the mutagenesis products confirmed that the desired mutant fragments had been successfully generated (data not shown). Equimolar quantities of reference wild-type PCR product and mutant PCR product were mixed, reannealed, and analyzed by DHPLC. Representative DHPLC elution profiles are shown in Figure 3. The chromatograms of the PCR products generated by site-directed mutagenesis exhibited multiple peaks, whereas those of the PCR products amplified from known wild-type homozygotes exhibited a sharp, single peak.

DISCUSSION

In the present study, we developed a DHPLC-based method allowing the entire coding region of the *JAG1* gene and the *NOTCH2* gene to be screened for point mutations, and small deletions and insertions. *JAG1* mutations were identified in more than two-thirds (13 out of 18) of the patients who had been clinically diagnosed as having the Alagille syndrome. In the remaining five patients, all the coding exons of *JAG1* were amplified by PCR and then sequenced in parallel, but no pathogenic mutations were identified. Subsequent analysis of the *NOTCH2* gene in these five patients using both DHPLC screening and complete sequencing of the coding exons failed to identify any mutations. Hence, the negative results for the DHPLC mutation screening for the *NOTCH2* gene were validated. The ability of the DHPLC-based method to detect all previously described *NOTCH2* mutant alleles generated by site-directed mutagenesis supports the notion that the DHPLC protocol described herein is a sensitive method, even if we did not identify any Alagille syndrome patients with *NOTCH2* mutations in the present study.

In 5 of the 18 patients we did not find a micromutation using DHPLC. Several factors may be responsible for a detection rate of less than 100%. First, some patients may have large deletions spanning one or several exons or even the entire *JAG1* and *NOTCH2* locus, which would be undetectable using our methodology. Large deletions can be detected on a DHPLC platform using a recently developed method called multiplex PCR/liquid chromatography assay (Dehainault *et al.* 2004; Udaka *et al.* 2006a, 2006b). A multiplex PCR with unlabeled primers enables simultaneous amplification of multiple exons under semiquantitative conditions and the PCR products separated by DHPLC are quantitated by fluorescence detection. Second, these patients may have mutations deep within introns or in other parts of the *JAG1* and *NOTCH2* gene that were not studied, such as the promoter. Finally, some patients enrolled in the present study may have a different condition that resembles Alagille syndrome such as Byler disease and other hereditary forms of intrahepatic cholestasis (Bull 2002).

The use of the COPPER plate (Kosaki *et al.* 2005; Udaka *et al.* 2005b; Aramaki *et al.* 2006; Udaka *et al.* 2006a) enables all the exons to be simultaneously amplified on a 96-well format PCR plate under the same cycling conditions, while the use of the computer script enables a completely automated DHPLC analysis of all the exons. These two features minimize the labor required by laboratory workers.

The implementation of the screening method for *JAG1* described herein will help medical geneticists confirm their clinical impressions. In the present study, eight patients had undergone liver biopsy before the diagnosis was confirmed by genetic testing. Since liver biopsy is a relatively invasive procedure when compared with genetic testing which is minimally invasive, we suggest that wider availability of the genetic screening test, as exemplified in the present study, would be beneficial to the Alagille patients and their families.

Precise documentation of mutations has clinical ramifications in genetic counseling of Alagille syndrome patients and their families. It is important to note that gonadal mosaicism has been documented in this syndrome (Giannakudis *et al.* 2001). Lack of the mutant allele in the parents' peripheral blood or father's sperm will be informative for the parents in terms of a low recurrence risk (Niu *et al.* 2006). The possibility of prenatal diagnosis in subsequent pregnancies could further reassure the parents.

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Research Letter

Two Distinctive Mechanisms Leading to Disruption of the *SHOX* Transcription Unit in a Single Family

Kosuke Izumi,[1] Masao Nakano,[2] Keisuke Kosaki,[3] Rika Kosaki,[4] Noboru Hosogai,[5] Hideo Matsumoto,[5] Tomonobu Hasegawa,[1] Takao Takahashi,[1] and Kenjiro Kosaki[1]*

[1]Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan
[2]Department of Obstetrics and Gynecology, Saiseikai Kanagawaken Hospital, Yokohama, Japan
[3]Department of Orthopedic Surgery, Tokyo Metropolitan Kita Medical & Rehabilitaion Center for the Handicapped, Tokyo, Japan
[4]Department of Clinical Genetics and Molecular Medicine, National Center for Child Health and Development, Tokyo, Japan
[5]Research and Development Division, Mitsubishi Chemical Medicine Corporation, Tokyo, Japan

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To the Editor:

Leri-Weill dyschondrosteosis (LWD) is a dominantly inherited form of skeletal dysplasia characterized by short stature and mesomelia accompanied by Madelung wrist deformity. The molecular basis of LWD is haploinsufficiency for the *SHOX* gene, which is located on the distal part of the X chromosome pseudoautosomal region (PAR1) [Belin et al., 1998; Shears et al., 1998]. About 60% of LWD patients have been reported to have *SHOX* mutations, including whole gene deletions [Falcinelli et al., 2002; Flanagan et al., 2002]. Benito-Sanz et al. [2005] reported 12 LWD patients who had heterozygous deletions of a putative *SHOX* enhancer region downstream of *SHOX*, without any alteration of the coding region of the *SHOX* gene itself. Subsequently, Fukami et al. [2006] identified an LWD patient with a comparable deletion and documented an enhancer element within the deleted segment using an in vitro experiment. Here, we describe a family in which two distinctive mechanisms led to the disruption of the *SHOX* transcription unit.

The propostia was referred to us during her fourth pregnancy because of a past history of multiple abnormal pregnancies. An amniocentesis was performed during her first pregnancy, and fetal amniocyte karyotyping performed by a commercial laboratory reported a 4p deletion. After genetic counseling, the parents elected to terminate the pregnancy. The second and third pregnancies ended in spontaneous abortions at 8 and 9 weeks of gestation, respectively. During the fourth pregnancy, fetal amniocyte karyotyping performed at another commercial laboratory reported a 46,X,del(X)(p22.1) karyotype.

At age 40 years, the proposita had a short stature (146 cm, below the 3rd centile, −2.4 SD), short arms, and bilateral Madelung deformities (Fig. 1A). A skeletal survey revealed short forearms with bowing of the radius and altered osseous alignment at the wrists (Fig. 1B). She was clinically diagnosed to have LWD. The proposita's sister, father, and paternal grandmother were reportedly short and had "bent-and-short forearms"; however, we did not have the opportunity to evaluate her relatives.

Her history of having fetuses with 4p monosomy and Xp monosomy prompted us to reevaluate the fetal karyotype using a 4p subtelomere fluorescence in situ hybridization (FISH) probe and an Xp subtelomere FISH probe (To TelVysion Multi-color FISH Probe Panel; VYS-33-270000). The fetus of the fourth pregnancy was subsequently found to have a derivative chromosome X in which Xp had been deleted and 4p had been translocated. G-banding studies and FISH analyses of the proposita's (i.e., mother's) peripheral blood revealed that she had a balanced translocation between 4p and Xp: 46,X,t(X;4)(p22.1;p16). Her husband had a normal karyotype. The karyotype of the fetus was reassigned

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*Correspondence to: Kenjiro Kosaki, Department of Pediatrics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan. E-mail: kkosaki@sc.itc.keio.ac.jp
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Address reprint requests to:
Kenjiro Kosaki, M.D., Ph.D., F.A.C.M.G.
Department of Pedaitrics
Keio University School of Medicine
35 Shinanomachi
Shinjuku-ku, Tokyo 160-8582, Japan

E-mail: kkosaki@sc.itc.keio.ac.jp

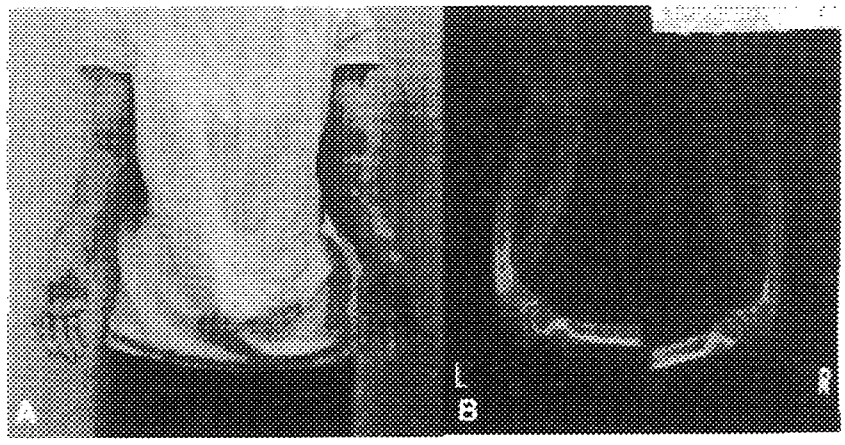

FIG. 1. Forearm of the proposita. **A**: Note Madelung deformity with bilateral cubitus valgus. **B**: Roentgenograms. Note curvature of radius and ulnar bones.

as 46,X,der(X)t(X;4)(p22.1;p16)mat. The parents elected to continue the pregnancy.

To further analyze the pathogenetic basis of the LWD phenotype in the mother, we performed a FISH study using the *SHOX* (Xp22.3) probe, as described by Ogata et al. [2001]. One *SHOX* signal was found on Xp, whereas the other signal was translocated on 4p (Fig. 2A). We performed additional FISH analyses using two long-range PCR products, one amplified from a genomic region 3′ to the *SHOX* 3′UTR (base position coordinates on chromosome X from Xpter according to the March 2006 human reference sequence NCBI Build 36.1 assembled by UC Santa Cruz: 541493–547861) and the other amplified from a genomic region 5′ to the *SHOX* 5′UTR (base position coordinates: 498463–504633). Both probes yielded a signal on derivative chromosome 4 (data not shown). We thus inferred that the entire *SHOX*-coding region was present on the derivative chromosome and that a deletion of *SHOX* per se was an unlikely explanation for the mother's LWD phenotype.

We first postulated that the mother's LWD phenotype would have been attributable to the splitting of the *SHOX*-coding region on the derivative chromosome 4 and the enhancer region on the derivative

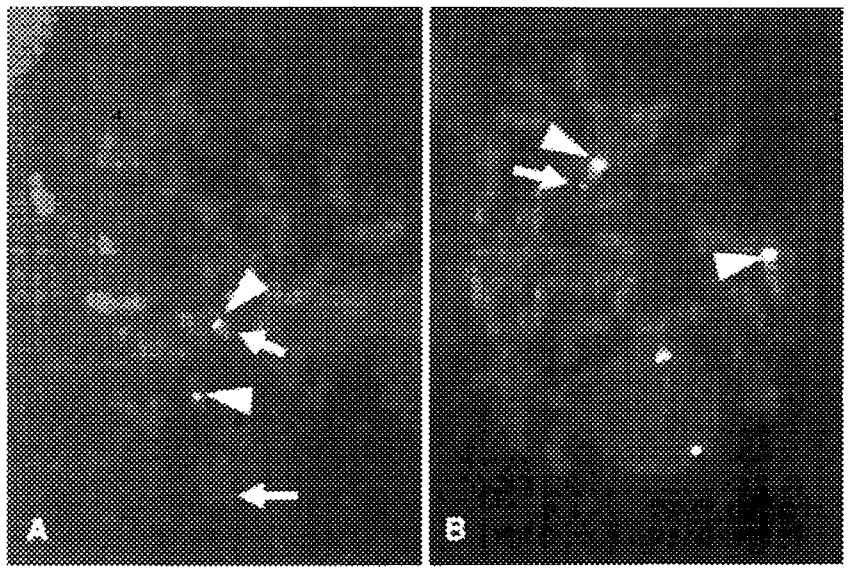

FIG. 2. Submicroscopic deletion of *SHOX* enhancer locus of the proposita. **A**: Fluorescence in situ hybridization (FISH) analysis with commercially available *SHOX* probe on metaphase spreads does not reveal a deletion of *SHOX* gene. Each two signals of *SHOX* (Xp22.3) probe (red signals, arrows) and chromosome X centromere probe, *DXZ1* probe (green signals, arrowheads) are depicted. One of the *SHOX* signal is detected on chromosome 4 due to the chromosomal translocation. **B**: FISH analysis with bacterial artificial chromosome (BAC) probe RPCI-11-946P8 containing the *SHOX* enhancer region. FISH analysis on metaphase spreads showing a deletion in one of the chromosome X homologs when hybridized with BAC RPCI-11-946P8 probes (red signal, arrow) and *DXZ1* probes (green signals, arrowheads).

chromosome X. We performed a FISH analysis using the bacterial artificial chromosome (BAC) probe RPCI-11-946P8, which spans the *SHOX* enhancer identified by Fukami et al. [2006]. Contrary to our prediction, only one copy of the enhancer was detected on the normal chromosome X (Fig. 2B). Hence, her LWD phenotype was ascribed to a deletion of the *SHOX* enhancer. The extent of the deletion flanking the *SHOX* enhancer was less than 1 Mb because an array CGH analysis (Spectral Chip 2600™; average resolution of 1 Mb) did not reveal any abnormalities [Cai et al., 2002]. We performed further analyses using two additional BAC clones, RPCI11-1119O18 and RPCI11-800K15, adjacent to the BAC clone RPCI11-946P8 that covers the enhancer region. The base position coordinates of RPCI11-946P8 on the X chromosome from Xpter, according to the March 2006 human reference sequence NCBI Build 36.1, are 693506–920272. FISH analysis using the BAC clone RPCI11-1119O18, with base position coordinates of 1141608–1338529, yielded two signals (data not shown). Hence, the centromeric end of the deleted segment was mapped between RPCI11-946P8 and RPCI11-1119O18. FISH analysis using the BAC clone RPCI11-800K15, with base position coordinates of 483105–664235, also yielded two signals (data not shown). Hence, the telomeric end of the deleted segment was mapped between RPCI11-946P8 and RPCI11-800K15.

The mother gave birth to her daughter at 39 weeks of gestation. The weight, height, and OFC of the daughter at birth were 3,017 g (50th centile), 49.0 cm (50th centile), and 33.0 cm (50th centile), respectively. At age 17 months, her height and weight were 77.1 cm (10–25th centile) and 11.05 kg (75–90th centile), respectively. Physical examinations at birth and at age 17 months did not reveal any dysmorphic features suggestive of Turner syndrome or 4p trisomy syndrome (Fig. 3). Radiography of her forearm revealed no deformity at 11 months.

The daughter exhibited normal to borderline psychomotor development; she smiled responsively and followed objects with eyes at age 3 months, gained head control at 4 months, and was able to transfer objects and sit by herself at 7 months, and stand with support at 11 months. She had not acquired any meaningful words at 14 months. G-banding karyotyping of the postnatal peripheral blood confirmed the results of the amniocyte karyotyping: 46,X,der(X)t(X;4)(p22.1;p16)mat.ish der(X)(tel 4p+,DXZ1+). The unbalanced karyotype presumably derived from the segregation of the derivative chromosome X and an intact chromosome during the maternal meiosis. FISH analysis using the *SHOX* (Xp22.3) probe did not show any signal on the derivative chromosome X (data not shown). The daughter was diagnosed as having monosomy Xp involving the *SHOX* locus and partial trisomy 4p.

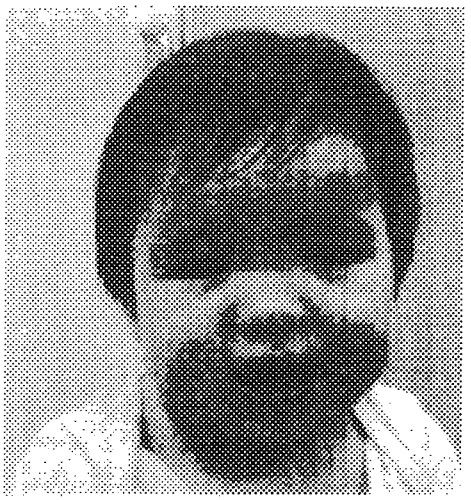

FIG. 3. The daughter at 17 months of age. Note mild frontal bossing and midface hypoplasia.

To further characterize the daughter's chromosomal dosage imbalance, an array CGH analysis was performed. A loss of signals was present from Xpter to Xp22.1, and the breakpoint on the X chromosome was localized between RPCI11-79B3 and RPCI11-487M22 (Xp22.1-Xp22.1); in contrast, a gain of signals was present from 4pter to 4p15.3, and the breakpoint on 4p was located around RPCI11-34C20 (4p15.33) (Fig. 4). According to the most recent human genome map (March 2006 human reference sequence NCBI Build 36.1), the *SHOX* enhancer and the translocation breakpoint on the X chromosome, as demonstrated by array CGH analysis, were 25 Mb apart.

The inactivation patterns of the X chromosomes in the proposita and her daughter were analyzed using genomic DNA from the mother, the daughter, and the proposita's husband and highly polymorphic repeats in the androgen receptor gene [Allen et al., 1992] or the *ZNF261* gene [Beever et al., 2003], which are adjacent to several CpG methylation-sensitive restriction enzyme sites that are differentially methylated on active and inactive X chromosomes.

The results from the androgen receptor gene were uninformative. The results from the *ZNF261* gene indicated that both the mother and the daughter had completely skewed X-inactivation. In the genomic DNA of the daughter, who had the unbalanced translocation, the maternal chromosome (the derivative X) was inactivated. In the genomic DNA of the mother, we were unable to determine whether the derivative X chromosome or the normal X chromosome was inactivated because samples of the proposita's parental genomic DNA were not available.

The mother manifested a complete LWD phenotype including short stature and Madelung deformity. Documentation of the mother, who exhibited a *SHOX*

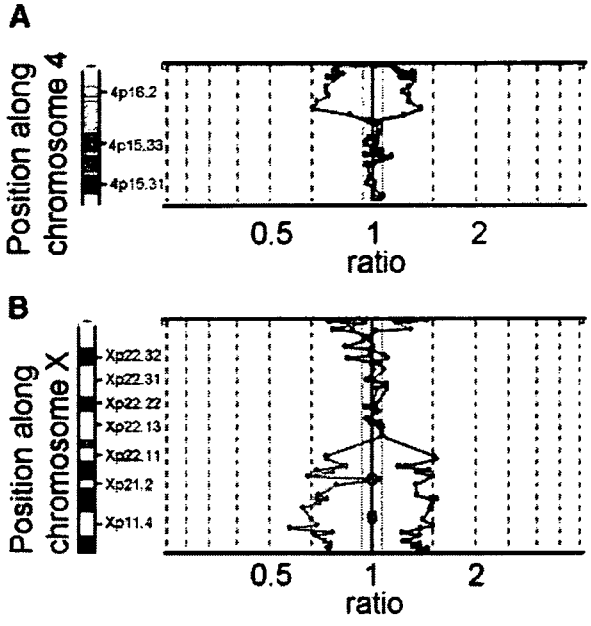

FIG. 4. Array CGH analysis of the daughter. Normalized copy number variation on BAC clones from chromosome 4 and X. Blue plots represent the patient-to-control (Cy5-to-Cy3) fluorescence intensity ratios for each clone. Red plots represent the result of dye swap, which was used to reduce the possibility of dye-related artifacts. Clones were considered deleted if the ratio of blue plot was <0.934 and ratio of red plot was >1.07, while a duplication was suspected if the ratio of blue plot was >1.07 and ratio of red plot was <0.934 except for the sex chromosomes, because sex mismatch controls was used. **A**: A plot for chromosome 4. Gain of 4p from 4pter to 4p15.3 (17 BAC clones) is demonstrated. **B**: A plot for chromosome X. Loss of Xp from Xpter to Xp22.1 (27 BAC clones) is demonstrated. The collapse of the X plot to normal from Xp22.3 to Xp22.1 shows loss of X in that area. Also, the loss of material from the Xpter to Xp22.3 (the pseudo-autosomal region) is consistent with the loss of that area.

enhancer deletion and intact coding region and who presented with the classic phenotypic features of LWD, further supports the recent observation that the deletion of a long-range *cis*-acting enhancer element constitutes an important etiology in LWD [Benito-Sanz et al., 2005; Fukami et al., 2006], as recently exemplified in other congenital disorders including camptomelic dysplasia with *SOX9* enhancer deletions [Wunderle et al., 1998; Pop et al., 2004], aniridia with *PAX6* enhancer deletions [Crolla et al., 1996; Lauderdale et al., 2000; Crolla and van Heyningen, 2002], and blepharophimosis syndrome with *FOXL2* enhancer deletions [Beysen et al., 2005]. The previously reported cases of *SHOX* enhancer deletions were not accompanied by translocation. Thus, the mother is unique in that the *SHOX*-coding region was translocated onto the derivative chromosome 4.

The chromosomal breakpoint on the X chromosome of the mother could be deduced from the daughter's array CGH results. The array-CGH analysis detected a terminal gain on 4p with a size of 11 Mb in addition to a terminal deletion on Xp with a size of 25 Mb and the location of the microdeletion containing the *SHOX* enhancer region and the translocation breakpoint was 25 Mb apart. No other deletions were detectable within this 25 Mb region, as shown by array CGH. Hence, the microdeletion of the *SHOX* enhancer and the X;4 translocation were physically separated. It is unclear whether the microdeletion event and the translocation event occurred in a mechanistically related fashion. A previous report of a comparable case in which an apparently balanced translocation was accompanied by a juxtaposed microdeletion suggests a possible causal relationship [Yue et al., 2005].

In contrast with the mother, the 17-month-old daughter with partial Xp monosomy and 4p trisomy manifested no apparent features of LWD. Because she has a partial Xp monosomy, we expect to observe Madelung deformity, an age-dependent feature that is not expressed before the onset of puberty, later on [Kosho et al., 1999]. When her karyotype was obtained prenatally, it was difficult to counsel the parents in regard to the possible developmental outcome of a 4p partial trisomy. Patel et al. [1995] reported that all 4p trisomy syndrome patients presented with moderate to severe developmental delays in their extensive review of more than 75 cases of patients with 4p trisomy. On the other hand, three patients with Xp/4p translocations had borderline to normal development: a 9-year-old female with der(X) t(X;4)(p21.2;p13) [Morichon-Delvallez et al., 1982], and a 50-year-old mother and a 17-year-old daughter with der(X),t(X;4)(p22.1;p14) [Petit et al., 1994]. These previous authors hypothesized that the effect of the autosomal 4p segment of the der (X) was modified by X-inactivation. The preferential selection of cells inactivating the derivative X chromosome over those inactivating the normal X chromosome, as documented by the methlyation analysis in the present study, might have partially alleviated the detrimental effects of partial 4p trisomy [Sharp et al., 2001, 2002]. As of the age of 17 months, the daughter had reached most developmental milestones almost on time except for language skill. Other than the lack of Madelung deformity, no discernible differences between the phenotypes of the mother and the daughter were noted.

In summary, we have described two distinctive mechanisms leading to the disruption of the *SHOX* transcription unit in a single family: the mother had a *SHOX* enhancer deletion with intact coding region, whereas the daughter had a deletion of the entire transcription unit. The documentation of this unique family illustrates the importance of detailed molecular cytogenetic studies when performing genetic counseling for apparently classic malformation syndromes.

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Screening for Alagille Syndrome Mutations in the *JAG1* and *NOTCH2* Genes Using Denaturing High-performance Liquid Chromatography

HAZUKI SAMEJIMA,[1] CHIHARU TORII,[1] RIKA KOSAKI,[2] KENJI KUROSAWA,[3] HIROSHI YOSHIHASHI,[3] KOJI MUROYA,[4] NOBUHIKO OKAMOTO,[5] YORIKO WATANABE,[6] TOMOKI KOSHO,[7] MICHIRU KUBOTA,[8] OSAMU MATSUDA,[9] MIWA GOTO,[1] KOSUKE IZUMI,[1] TAKAO TAKAHASHI,[1] and KENJIRO KOSAKI[1]

ABSTRACT

Mutations in the *JAG1* gene and the *NOTCH2* gene cause Alagille syndrome. At present, however, genetic testing of Alagille syndrome is not commonly applied in clinical settings because the currently available assays are technically and financially demanding, mainly because of the size of the genes. In the present study, we optimized the highly sensitive and specific mutation scanning method automated denaturing high-performance liquid chromatography (DHPLC) to analyze the entire coding region of *JAG1* and *NOTCH2*. The coding region was amplified by 69 primer pairs, all of which have the same cycling conditions, aliquoted on a 96-well format PCR plate. In this manner, all the exons were simultaneously amplified using a single block in a thermal cycler. We then wrote a computer script to analyze each segment of *JAG1* and *NOTCH2* by DHPLC in a serial manner using conditions that were optimized for each amplicon. The implementation of this screening method for *JAG1* and *NOTCH2* will help medical geneticists confirm their clinical impressions and provide accurate genetic counseling to the patients with Alagille syndrome and their families.

INTRODUCTION

ALAGILLE SYNDROME (MIM 118450) represents an autosomal dominant condition characterized by a constellation of malformations: a paucity of intrahepatic bile ducts, posterior embryotoxon, and retinal pigmentary changes in the eye, pulmonic valvular stenosis and peripheral arterial stenosis in the heart; abnormal vertebrae ("butterfly" vertebrae), and decrease in interpediculate distance in the lumbar spine in the skeletal system; broad forehead, pointed mandible, and bulbous tip of the nose (Watson and Miller 1973;Alagille *et al.* 1975), and renal dysplasia (LaBrecque *et al.* 1982).

Mutations in the *Jagged 1* gene (*JAG1*) on chromosome 20p11.2 were identified as causative of this syndrome in 1997 (Li *et al.* 1997; Oda *et al.* 1997). *JAG1* encodes a transmembrane protein acting as ligand for the evolutionarily conserved Notch signaling pathway. Since then, more than 24 mutation analyses have been published regarding this locus. In 2006, *NOTCH2*, a notch signaling receptor, was identified as the second causative gene for Alagille syndrome (McDaniell *et al.* 2006). Among 11 *JAG1* mutation-negative probands, two probands with Alagille syndrome were identified as having *NOTCH2* mutations. The 11 patients derived from 247 Alagille probands. Hence, only a small fraction (*i.e.*, 2/247) of patients with Alagille syndrome had *NOTCH2* mutations.

AU1 [1]Department of Pediatrics, Keio University School of Medicine, [2]Department of Clinical Genetics and Molecular Medicine, National Children's Medical Center, Divisions of [3]Medical Genetics and [4]Endocrinology, Kanagawa Children's Medical Center, Tokyo, Japan.
[5]Department of Planning and Research, Osaka Medical Center and Research Institute for Maternal and Child Health, [6]Department of Pediatrics and Child Health, Kurume University School of Medicine.
[7]Department of Medical Genetics, Shinshu University School of Medicine.
[8]Department of Pediatrics, Hokkaido University School of Medicine.
[9]Department of Pediatrics, Ehime University School of Medicine.

Alagille syndrome is characterized by extreme degree of variable expressivity and many atypical or mild cases have been reported (Kamath *et al.* 2003). Clinical diagnosis can be difficult in these cases, and sometimes liver biopsy is performed to search for paucity of intrahepatic bile duct, and molecular diagnosis represents a noninvasive alternative option to liver biopsy. Usefulness of the genetic test is exemplified in cases in which liver or kidney transplantation is undertaken. When severe hepatic (Kasahara *et al.* 2003) or renal failure (Schonck *et al.* 1998) occur in an Alagille syndrome patient, liver or kidney transplantation is performed. Occasionally, a graft is obtained from a living related donor (Kasahara *et al.* 2003), typically from either of the parents. As stated above, because of extreme degree of variable expressivity, potential donors can exhibit minimal clinical phenotype yet carries the same mutation as the patient (*i.e.*, the recipient). In order to prevent those asymptomatic carriers from being donors, liver biopsy may be performed. Molecular diagnosis can alleviate such invasive tests. At present, however, genetic testing of *JAG1* is not commonly applied in clinical settings because the currently available assays are technically and financially demanding, mainly because of the size of the *JAG1* gene, which comprises a coding sequence of 3657 bp in 26 exons. Since mutations in the second causative gene *NOTCH2* cause comparable phenotype, *NOTCH2*, which comprises a coding sequence of 7416 bp in 34 exons, needs to be screened for when no mutation is identified in *JAG1*. This requirement further increases the number of exons to be screened.

Generally speaking, two approaches can be taken when performing genetic testing in clinical settings. Most intuitively, all the coding exons can be amplified by PCR and then sequenced in parallel. Alternatively, screening procedures can be performed prior to the sequencing analysis to identify exon(s) that may harbor mutations. When the size of the gene (*i.e.*, the number of exons) is small, the first approach is more efficient. However, as the size of the gene increases, the second approach becomes more efficient from the standpoints of supply costs and labor. Because the human *JAG1* gene and the *NOTCH2* gene are relatively large, consisting of 60 exons altogether, clinical genetic testing of Alagille syndrome is best accomplished by the second method. Heritage *et al.* (2002) demonstrated that denaturing high-performance liquid chromatography (DHPLC) technique (O'Donovan *et al.* 1998; Wagner *et al.* 1999; Udaka *et al.* 2005b) is suitable for such screening. In the present study, we developed DHPLC-based systematic mutation screening system for semiautomated analysis of multiple exons of *JAG1* and *NOTCH2* (Kosaki *et al.* 2005), which requires minimal human intervention, with a consequent significant reduction of the labor cost (Aramaki *et al.* 2006). This article presents in detail the methods we have developed for finding mutations in the *JAG1* and *NOTCH2* genes, which cause the majority of cases of the Alagille syndrome.

MATERIALS AND METHODS

Patients and DNA preparation

Eighteen Japanese probands who had been clinically diagnosed as having Alagille syndrome (Alagille *et al.* 1987) were included in the study. All the patients *except for two* were simplex (*i.e.*, a single occurrence in a family). In families with multiple affected family members, only the probands were included. Each patient and family members were enrolled in the study after receiving their written informed consent, according to a protocol approved by an institutional review board. Genomic DNA was isolated using the QIAamp system (Qiagen Inc., Valencia, CA).

PCR amplification of the genomic DNA

The entire *JAG1* coding region (exons 1–26; GenBank sequence accession number NM_000214) was screened for mutations. We carefully selected the primer pairs to ensure that all of the primers could use the same cycling conditions. The primer pairs were designed to amplify the exons, exon–intron boundaries and at least 20 base pairs of the flanking intron sequences. Twelve of the 60 primers had been used in a previously published study (Oda *et al.* 1997). The remaining 48 (60–12) primers were designed by us. The optimized primer sequences are listed in Table 1.

PCR was performed in a volume of 20 μl containing 30 ng of genomic DNA, 10 pmol each of the forward and reverse primers, 0.2 m*M* of each dNTP, 2 m*M* $MgSO_4$, 0.5 units of Platinum *Taq* DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA), and the buffer supplied by the manufacturer. Thermal cycling was done using a touchdown PCR protocol (Don *et al.* 1991); The annealing temperature was decreased by 0.5°C every second cycle beginning at 65°C and decreasing to a "touchdown" annealing temperature of 60°C, which was then used for 30 cycles.

JAG1 *mutation analysis*

PCR amplicons from human genomic DNA were analyzed by DHPLC according to the method developed by Oefner and Underhill (O'Donovan *et al.* 1998; Wagner *et al.* 1999; Udaka *et al.* 2005a) using an analysis system purchased from Transgenomic (Omaha, NE). To generate heteroduplexes, the PCR products were denatured at 95°C for 5 min and reannealed by cooling to 25°C with a temperature change of −1.5°C/min. After heteroduplex formation, the PCR amplicons were applied to a preheated reversed-phase column. An elution gradient was generated by mixing buffer A (0.1 mol/l triethylammonium acetate) and buffer B (0.1 mol/l triethylammonium acetate containing 250 ml/l acetonitrile) in a linear gradient from start to a final %B over a period of 4.5 min, as described in Table 1. Standard operating procedures for the instrument's operation and maintenance and for mutation detection by DHPLC (http://cmgs.org/bpg/Guidelines/2002/dhplc.htm) have been described (Schollen *et al.* 2005).

All DHPLC conditions, including the melting temperatures and buffer gradients specific to each PCR amplicon, were determined using melting temperature prediction software (Transgenomic WAVEMAKER). Multiple column temperatures were used when the software predicted that the fragment being analyzed consists of two or three different melting temperature domains. In that case, two or three analysis temperatures were needed to scan the entire exon sequence. Two or three temperature conditions were used for each amplicon, except for exons 2a, 2b, 3, 4b, 14, 17, 18, 19, 22, and 26c, which

TABLE 1. PCR PRIMER SEQUENCES AND DHPLC ANALYSIS CONDITIONS (TEMPERATURE AND GRADIENT) FOR *JAG1* MUTATION SCREENING

Exon[a]	*F/R*[b]	*Primer sequence (5'-3')*	*DHPLC temp (°C)*	*DHPLC gradient*[c] *(% B/4.5 min)*
1	F	TCCAATCGGCGGAGTATATTAGAGC[d]	66.2	52–61
	R	AGGACGGCTGGGAGGGA[d]	70.5	47–56
2a	F	GCTGACCTACCTCCTTCCCT	64.6	54–63
	R	GATGCGGTTGCGGTCGTTG		
2b	F	TCATCGGGGGCAACACCTTC	66.7	46–55
	R	CAGGCGCGGGTGTGAG		
3	F	TGACAAAGGAAGGGGAGTTGGTTTG	59.4	51–60
	R	GTTTAGAGAAAAGTCCACAGAAGCGATAC		
4a	F	TCAGGGAAGAAGGCTGCAATGTGAATA	57.6	51–60
	R	CTGGGGCGGCAGAACTTATTG[d]	63.6	45–54
4b	F	GCGTTGCCCQACTTTGAGTAT	60.9	52–61
	R	TGGTGGGGTGATAAATGGAC		
5	F	GCGTCCCGATGCAAGGAACAGGCAGTGT	57.2	53–62
	R	AGAAGTGGTGTGTGCATTCC	60.1	50–59
6	F	CAAAAGGCTAACCTGGAGGTGTG	62.7	50–59
	R	GATCCCACCCTGGGTCTCAT	63.7	49–58
7	F	TGGGTTCGCCATCTTCACAGG[d]	58.9	52–61
	R	CGAATCAGCATCACCCAAAAAACTTTAGAGA	60.0	51–60
8	F	ATCCCTCTCTGCTGCCATCC	60.4	51–60
	R	CTCTCCCCAGCTGGTATCTT	62.7	49–58
9	F	GGCCCGCCGCCCCCGCCGGTCAACCCCCTCCTTTTCTT	59.8	51–60
	R	CAATCAAAGCCAACCTTGGT6A	61.5	50–59
10	F	ATTTCTCATGCTCATCCCCATCTCCTT	58.6	50–59
	R	CAGCAAGTCGGCTACCCAAGTTT[d]	60.6	48–57
11	F	CCCAATTTCACTGTAAATTACCTCTTTAAAATGATGAC	53.3	49–58
	R	AGCTCTCCTAGTGTCGCACAAATCT		
12	F	CTGAAGCCCTGTGTTTGTGGAATAC	58.8	55–64
	R	GAAAAGTAAAGGGAAGCGGAGGAG[d]	60.5	53–62
13	F	CCCTCCCCCTTTTCGCTGTT[d]	60.2	52–61
	R	TAGTAAGTGGGGACAAAAGGAGCAAGT	62.7	50–59
14	F	GAATGCCGCATCTGTGGGTG[d]	61.2	53–62
	R	GCTGGGGAGCACTGGTC		
15	F	AGGAGGGAGCCATGAAAACTGC[d]	58.1	52–61
	R	AACATGACCCATACATCCCAGAG	61.3	49–58
16	F	GTGAATGGTCCTGGATCTCGTCTT[d]	59.8	52–61
	R	CCCTCCCACAGAAGACAGAG	63.0	48–57
17	F	GCTATCTCTGGGACCCTTCC	61.4	54–63
	R	CCAGGCCCAGAGAAATATCA		
18	F	TTATGATATTTCTCTGGGCCTGGTTCTTG	62.8	51–60
	R	CGACAGCCCTGGGAGAGTT		

TABLE 1. PCR PRIMER SEQUENCES AND DHPLC ANALYSIS CONDITIONS (TEMPERATURE AND GRADIENT) FOR *JAG1* MUTATION SCREENING (CONT'D)

Exon[a]	*F/R*[b]	*Primer sequence (5'-3')*	*DHPLC temp (°C)*	*DHPLC gradient*[c] *(% B/4.5 min)*
19	F	GCCTGGTTCCAATTTAGCAA	59.7	49–58
	R	GCGTCCCGCACGATAGTGGATGAGTGCTG		
20	F	AGAGTAATGGACTGGGAGGTTGGTAA[d]	60.3	50–59
	R	TGAGGCATGGAATGAAGCGGTAAAG	63.5	46–55
21	F	ACCATCAGTCCCTAAACTTGAACTCCATT	59.4	50–59
	R	GCTCACCCCAGAAGACCCAT	63.0	47–56
22	F	CAAAGGGCACAGGCATAACCATTTCATAA	61.1	51–60
	R	GTGAACTGCGGCAGCCATCAT	57.0	56–65
23	F	TTCTGGAATCAGGGATGAGC	61.0	52–61
	R	GCAGACATCCACCATTCAAA	64.5	48–57
24	F	CTCTCAATCTTACACGTGTGTGGGTTT	57.3	51–60
	R	CATCGAATAATGAGGTGTGAATGGGTC	60.3	48–57
25	F	TCGAGTAATTTTGAAGAAAGGCTGCTTTGAGTAT	57.9	53–62
	R	ATAATCCCTCGACCTGATGGCTTTATTGAA	59.9	51–60
26a	F	TTCTTTCTTTCTTGGAGAGTTAATTGGTTTTGTGC	60.3	53–62
	R	ATAATCCTTGATGGGGACCGTGTTG	64.4	49–58
26b	F	CAGCTGAACCAGATCAAAAACCCCA	58.7	52–61
	R	TGTTTGTCCAGTTTGGGTGTTTTGTCG	62.9	48–57
26c	F	GTACACGCTGGTAGACAGAGAAGA	61.9	50–59
	R	GACAGTTTAAAGAACTACAAGCCCTCAGA[d]		

[a]Some exons are amplified in multiple PCRs, designated a, b, etc., and some PCRs contain more than one exon.
[b]F, upstream primer; R, downstream primer.
[c]Buffer A is 0.1 *M* triethylammonium acetate; Buffer B is 0.1 *M* triethylammonium acetate containing 25% (v/v) acetonitrile. "52–61" indicates that the gradient consists of 52% B: 48% A through to 61% B: 39% A.
[d]Primers which had been used in a previously published study (Oda *et al.*, 1997).

were subjected to only a single condition. The optimized DHPLC conditions for each amplicon are listed in Table 1.

When the chromatographic analyses of all the amplicons were completed, the DHPLC profiles were visually compared with the profiles of normal controls. PCR products corresponding to all variant elution profiles of the DHPLC were purified using a desalting column and were sequenced bidirectionally using the dideoxy sequencing method (BigDye Dideoxy sequencing kit; Applied BioSystems, Foster City, CA) and an automated sequencer (ABI3100; Applied Biosystems). The sequence-verification primers were the same as the PCR primers.

NOTCH2 *mutation analysis*

We analyzed the entire coding region (exons 1–34) of the *NOTCH2* gene in five genetic samples from patients in whom no *JAG1* mutations had been identified. The entire *NOTCH2* coding region (exons 1–34; GenBank sequence accession number NM_024408) was screened for mutations. We carefully selected the primer pairs to ensure that all of the primers could use the same cycling conditions. All the primer pairs except for one (McDaniell *et al.* 2006) were designed by us. The optimized primer sequences are listed in Table 2. All DHPLC conditions, including the melting temperatures and buffer gradients specific to each PCR amplicon, were determined using melting temperature prediction software (Transgenomic WAVEMAKER) and optimized as described above. Two to four temperature conditions were used for each amplicon, except for exons 1, 2, 10, and 22, which were subjected to only a single condition. The optimized DHPLC conditions for each amplicon are listed in Table 2.

Site-directed mutagenesis of NOTCH2

In order to confirm that our method is adequate for the detection of *NOTCH2* mutations, we generated PCR amplicons containing all previously described mutant alleles of *NOTCH2*, 1331G>A(C444Y), and IVS32-1G>A , using the "splicing by overlap extension" (SOE) PCR method (Horton *et al.* 1990,

TABLE 2. PCR PRIMER SEQUENCES AND DHPLC ANALYSIS CONDITIONS (TEMPERATURE AND GRADIENT) FOR *NOTCH2* MUTATION SCREENING

Exon[a]	*F/R*[b]	*Primer sequence (5'-3')*	*DHPLC temp (°C)*	*DHPLC gradient*[c] *(% B/4.5 min)*
1	F	CACACGAGGCTGCTTCGT[d]	68.0	56–65
	R	CACACGAGGCTGCTTCGT[d]		
2	F	CTAAAACACAGGAAATAAGAGCATC	56.5	58–67
	R	TCTTATTAGCCAATTATCTGGCATC	58.5	55–64
3	F	TGTTTTCATGGACTCTTCTCTTTTC	61.5	53–62
	R	GGTATCTGCTGAAGGTAGGAAAC	64.0	50–59
4	F	CTATTTCTGTGGCCAGTACTGAGTT	60.8	58–67
	R	CTCACTTCCCTTTTTCCTTGG	62.8	56–65
5	F	CAGGTCGTTTCTTCTGTATGAGATT	59.5	51–60
	R	GCAGGCCTAAGATATTTGTTACTGA	61.2	49–58
6	F	GCTGGTATGGTACTAGTCCTTTGAG	59.6	55–64
	R	CCTGTTTCTAGATCCGTCCTTCTG	62.3	53–62
7	F	GAAGTTGCGTATAAATCTCCATGTT	59.4	56–65
	R	GGAATATATGGTTATGGCTATGCTG	60.8	53–62
8	F	ATGCTATAACCATCAGATGCTCTTC	58.4	55–64
	R	GAATGCTCTACCAAGAGAAGTTCAG	60.2	53–62
			61.0	53–62
9	F	TTCTCTCTCTTTAAGCCACTTGTGT	57.2	53–62
	R	TACTCTAATGATTTCCTCATGCACA	60.2	50–59
			61.3	49–58
10	F	CCTAACATCATTCTCATGGTAAAGG	59.5	54–63
	R	AGAAACAGCTCTTCCTAAACACAGA		
11	F	CTTGGTCTTTGAGAATCCTTGATAA	58.5	55–64
	R	ACAGATTTGAGAAACAGTGAAAACC	61.1	52–61
			62.3	51–60
12	F	CATCACAAGCAGAAAGCCTTAGTAT	57.4	52–61
	R	GAAATAGGAAACACTTGAQAAAGCAA	60.1	51–60
13	F	AGTTCTGGGTCTATTTCCTAAGCAT	60.2	53–62
	R	AACTGGCTTAACACAGGTCAATAAG	61.7	52–61
14	F	GATCAGTTTACCCCATTTCTTCAC	58.3	52–61
	R	AGAGCAGACGCAGAAAGATGTACT	59.5	51–60
			56.6	52–64
15	F	AATGCAATTTCTGATGTGTTCAGT	59.0	54–63
	R	CAAAGCTCAAGATCCAGTCAGTAA	62.0	51–60
16	F	TTTTCAAAGCCTCTTATCAGGAC	56.2	55–64
	R	GCCTTCCATATGATCTGATAACCT	59.2	52–61
17	F	AGCTTCCAAGGACTAGGTCATTTA	60.2	53–62
	R	AATATATTCCACTGCCTCAATTCC	61.0	53–62
18	F	AACTGATGCTACTCTTCAGAGGTCTA	58.7	55–64
	R	CTCTGAAATTTCCTTCCCTTTCTC	60.5	54–63
19	F	AGTAAGTCACAGGCACCAGATACA	59.4	54–63
	R	ACCATTTTGGAATCTGCAAGTTAT	60.4	53–62
			62.8	50–59
20	F	ATGTATGTACACCCCTGCCTCT	60.3	51–60
	R	TGATACCTTCTCTAAATGCCACTG	61.3	50–59
21	F	GGTTGTGAAGAACTGGCTAAGAAT	61.0	53–62
	R	AGGCTGAAAGAAACAGTGGTAAAC	63.0	51–60

TABLE 2. PCR PRIMER SEQUENCES AND DHPLC ANALYSIS CONDITIONS (TEMPERATURE AND GRADIENT) FOR *NOTCH2* MUTATION SCREENING (CONT'D)

Exon[a]	*F/R*[b]	*Primer sequence (5'-3')*	*DHPLC temp (°C)*	*DHPLC gradient*[c] *(% B/4.5 min)*
22	F	TGGTCCAATATTTCTTGTCCTTTT	61.1	49–58
	R	AAAATCCCCTGAACACTAAGAATG		
			59.8	55–64
23	F	GGATTTTCATAATTCTGTACCTCTTC	61.2	54–63
	R	CCAATTTGTTCACTAAAACCATCC	63.2	52–61
24	F	ATTACCACAGGCCTAGAACTGAAC	57.2	55–64
	R	CAGATTGTATGGAAGAGACAATGC	62.0	50–59
			61.9	55–64
25a	F	ATATGACTGTTTTCCTACCAATGC	63.8	54–63
	R	ACAATACTGGCTCAGACAGGTG	66.5	51–60
25b	F	CTCCTTATTACTCCTGCCAGTGTG	60.5	56–65
	R	GTTAGTGACAGTCCCCTTCAGTTC	62.5	54–63
			58.8	58–67
26	F	AAATGGGTAGGAAAATCCACAGTA	59.5	57–66
	R	GGCTTCATAAAATTAGCCTTGAA	61.2	55–64
			63.0	53–62
27	F	CTGACCTGCACTCTTCTGTTTTAC	60.8	53–62
	R	GATTCATTTAGAGGGCAACTTTGA	62.5	51–60
28	F	GGGAACAATTTTCATTATGTAGCC	59.2	54–63
	R	GAGTAAATGCCCAGAGCTTAGAAT	62.0	51–60
			55.8	53–62
29	F	CCAAGACCCTGCAAAATTGTAATA	57.8	51–60
	R	CAGATAATGGCTGACAATGGTG	60.2	49–58
30	F	AGATTGATAGGGAGCATTGTTTTC	59.4	55–64
	R	ATTCATTTCCTCGAGCTGATTTAG	63.0	52–61
31	F	GGGTTAACTACCTTCTCCTTCTTG	60.1	56–65
	R	CGATAAAACATTATAGAGCCATAGGA	63.5	53–62
			64.2	52–61
			58.8	53–62
32	F	TTCATTATTTTCCTTCATCCAGGT	61.2	51–60
	R	GTAACTCTATTCCCCTCGTCAGAG	63.5	49–58
33	F	CTGGGGAGTTTAAGATTTCATTGT	60.0	49–58
	R	CAAGAGAAGCTGTAAGGAGAAACG	61.0	48–57
34a	F	GTTATTAACATGTGTTCTGTGATGG	60.3	57–66
	R	CCTTCTCACTCAGAGACTTCTTCC	61.5	56–65
34b	F	AAGAAGTCTAGACGGCCCAGT	59.8	56–65
	R	ACACAATGTGGTGGTGGGATAG	62.0	54–63
34c	F	GCCCAGCATGCACTATCTTT	61.6	57–66
	R	CATTTCTGGAATCTGGTACATGGT	62.5	56–65
			61.6	57–66
34d	F	CTGAAGGGAAGCACATAACCAC	62.6	56–65
	R	CTGACCAGTCAGAAGCAGAGTG	64.5	54–63
			61.2	54–63
34e	F	AGCACAGTTATGCTTCCTCAAAT	62.2	53–62
	R	CAGCAGCATTTACAAAAGTCAGGTT	63.5	52–61

[a]Some exons are amplified in multiple PCRs, designated a, b, etc., and some PCRs contain more than one exon.
[b]F, upstream primer; R, downstream primer.
[c]Buffer A is 0.1 *M* triethylammonium acetate; Buffer B is 0.1 *M* triethylammonium acetate containing 25% (v/v) acetonitrile. "56–65" indicates that the gradient consists of 56% B: 44% A through to 65% B: 35% A.
[d]Primers which had been used in a previously published study (McDaniell *et al.*, 2006s).

TABLE 3. PCR PRIMER SEQUENCES FOR SITE-DIRECTED MUTAGENESIS OF PREVIOUSLY DESCRIBED MUTANT ALLELES OF *NOTCH2*

Mutant alleles	*Outer forward primer*[a]	*Inner reverse primer sequence (5′-3′)*[b]	*Inner forward primer sequence (5′-3′)*[b]	*Outer reverse primer*[a]
1331G > A (C444Y)	exon 8 F	ACCCTTCAGATACTCACAGTGG	CCACTGTGAGTATCTGAAGGGT	exon 8 R
IVS32-1G > A	exon 33F	GAAGAGCAGATTTTCTTGCAG	CTGCAAGAAAATCTGCTCTTC	exon 33 R

[a]See Table 2 for primer sequence.
[b]Underlined nucleotide shows mismatch position.

1993; Udaka *et al.* 2005b). Briefly, two segments of a gene were independently amplified by PCR and then fused together in a subsequent reaction. Mutations were introduced into a targeted region using mutant primers containing mismatches in their central region. Because the mutant primers were complementary, the two overlapping fragments could be fused together in a subsequent extension reaction. Two PCR steps were performed to amplify two separate products using appropriate outer and inner mutated primers. PCR conditions for the primary PCR were 30 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 60 sec using outer forward–inner reverse and inner forward–outer reverse primer pairs (Table 3), respectively. Then,

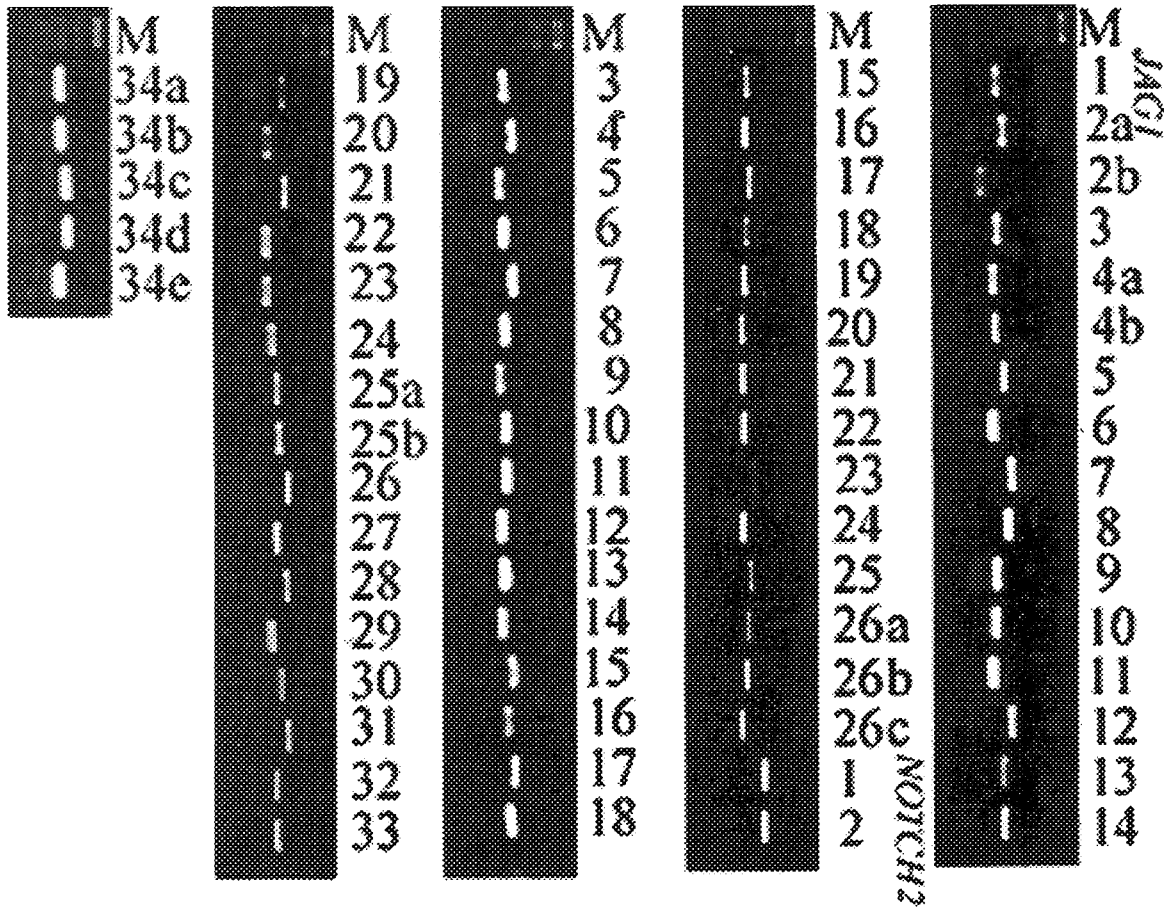

FIG. 1. PCR amplification for all coding exons of *JAG1* and *NOTCH2* using the same amplification conditions. One percent agarose gel electrophoresis of all 69 amplicons covering the entire coding regions of the two genes is shown. The leftmost lane (M) in each gel contains 1-kb ladder markers corresponding to 100-, 200-, 300-, 400-, 500-, 650-, 850-, and 1,000-bp fragments; the other lanes were loaded with 3 μL of PCR products. Please note that this figure was presented for illustrative purposes. We do not usually subject each plate to agarose gel electrophoresis prior to DHPLC analysis.

PCR products were purified by desalting column (Qiagen). Secondary PCR was performed using the outer forward and outer reverse primers and an appropriate amount of two primary PCR products as a template, using the same conditions as for the primary PCR. The PCR products were purified using a desalting column (Qiagen), sequence-verified, and used as the positive controls for the DHPLC assay.

RESULTS

Optimization of the PCR conditions

The coding regions of *JAG1* and *NOTCH2* were amplified in 30 and 39 amplicons, respectively (Tables 1 and 2). The 30 + 39 primer pairs, all with the same cycling conditions, were aliquoted on a 96-well format PCR plate. In this manner, all the exons were amplified simultaneously using a single block of a thermal cycler. The PCR plate will be referred to as the Condition-Oriented-PCR primer-Embedded-Reactor plate (COPPER plate) (Kosaki *et al.* 2005). All the exons were successfully amplified under a single condition without producing any artifacts from mispriming or primer–dimer formation (Fig. 1).

Optimization of the DHPLC conditions

The predicted optimal column temperatures and elution gradient for the DHPLC analysis of each PCR amplicon were verified by confirming that the elution profile of each of the PCR amplicons generated from wild-type genomic DNA (Kosaki *et al.* 2005; Udaka *et al.* 2005a, 2005b) had a sharp and solitary peak. The optimized column temperatures and elution gradients for the DHPLC analysis of each PCR amplicon are shown in Table 1. Figure 2 shows DHPLC profiles for the *JAG1* mutations discovered in our patient population.

Several specific factors are known to affect the sensitivity of the DHPLC assay. First, the use of impure oligonucleotide primers for PCR amplification can yield false-positive results. Second, poor quality of the DNA polymerase can also yield false-positive results (Kosaki *et al.* 2005). Third, the fragment size can affect the sensitivity of the assay; the optimal fragment size ranges from 150 bp to 700 bp (Xiao and Oefner 2001). Fourth, a high G-C content may affect the performance of the

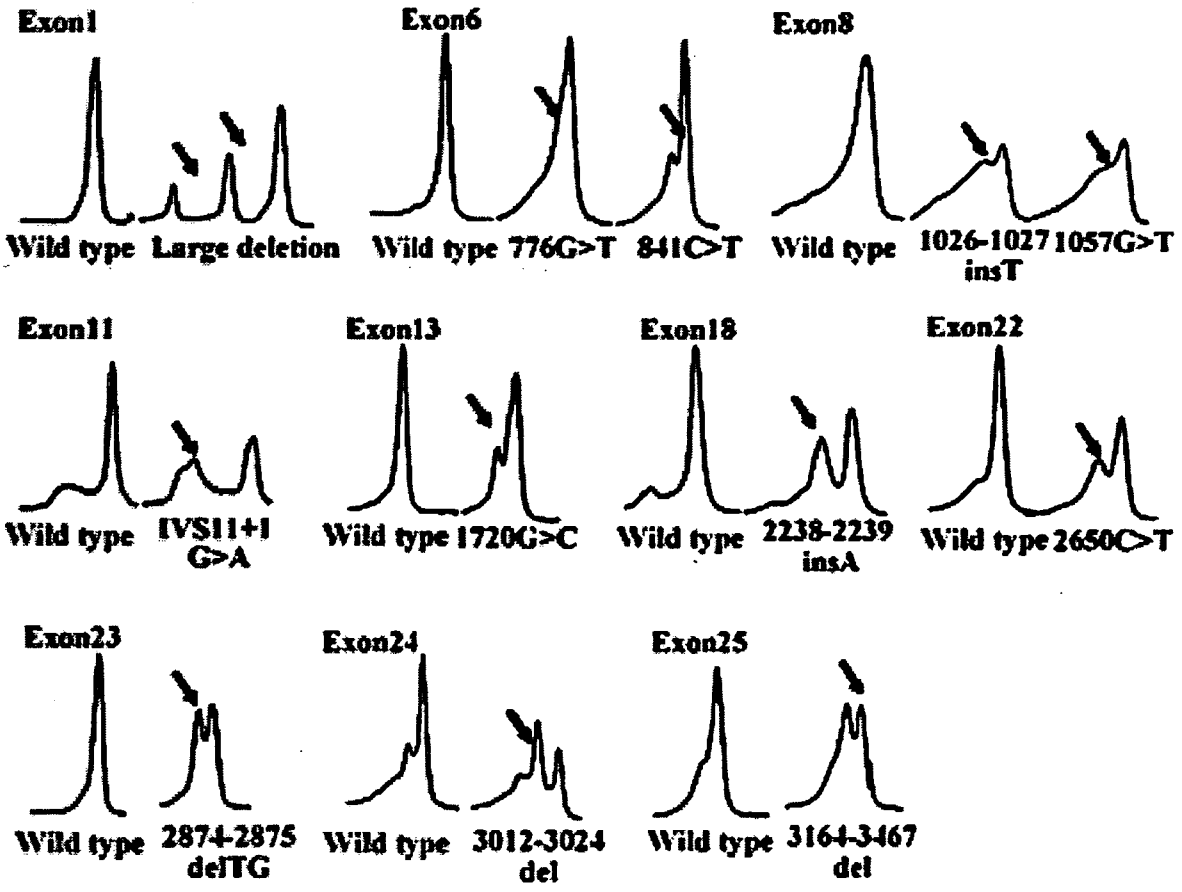

FIG. 2. DHPLC elution profiles of *JAG1* mutations detected in the present study. The column temperatures were 70.5°C, 62.7°C, 62.7°C, 53.3°C, 60.2°C, 62.8°C, 61.1°C, 61.2°C, 57.3°C, and 57.9°C for exons 1, 6, 8, 11, 13, 18, 22, 23, 24, and 25, respectively. Only the chromatogram with the best resolution of the heteroduplex peak is shown for each mutation.

TABLE 4. CLINICAL FEATURES OF THE PATIENTS WITH JAG1 MUTATION

Mutations		*Deletion including start codon*	*776G>T*	*841C>T*	*1026-10 27 insT*	*1057G>T*	*IVS11 +1G>A*	*IVS11 +1G>A*	*1720G> C*	*2238-223 9 ins A*	*2650C>T*	*2874–2875 delTG*	*3012-3024del TTCCCCTTC AGCG*	*3164–3167 delTAAG*
Location		*Exon 1*	*Exon 6*	*Exon 6*	*Exon 8*	*Exon 8*	*Intron 11*	*Intron 11*	*Exon 13*	*Exon 18*	*Exon 22*	*Exon 23*	*Exon 24*	*Exon 25*
Domain		*NAP*	*EGF1*	*EGF1*	*EGF4*	*EGF4*	*EGF10*	*EGF10*	*EGF14*	*CR*	*CR*	*C958 fs X965*	*P1004*	*V1055 fs*
Amino acid substitution		*NAP*	*G259V*	*Q281X*	*EGF4 D343X*	*E353X*	*NA*	*N.A.*	*V574L*	*S747 fs 755X*	*Q884X*		*fs1031X*	*X1061*
Sex	*Male/Female*	*F*	*M*	*M*	*F*	*F*	*M*	*F*	*F*		*F*	*M*	*M*	*F*
age	*y m*	*Died at 12 y*	*3y 11m*	*31y*		*11y 9m*	*1y 1m*	*5y*	*0y 5 m*		*0 y 5 m*	*12y*	*8 y 5 m*	*6 y 5 m*
Craniofacial	deep set eyes	NA.V	+	−	−	+	+	+	−	+	+	+	+	−
	retrognathia /prominent chin	NAV	+	+	−	+	+	−	−	+	+	−	+	+
Ophthalmic	posterior embryotoxon	−	+	+	−	−	−	+	−	−	+	−	+	+
	iris anomaly /axenfeldt anomaly	−	−	+	−	−	−	−	−	−	−	−	+	−
	retinal degeneration	−	−	+	−	−	+	−	−	+	−	−	+	−
Cardiovascular	peripheral pulmonary stenosis	−	+	−		+	+	−	−	+	+	+	+	+
	other congenital heart defects	ASD	TOF	−	−	−	−	ASD, PS	PS	−	−	−	TOF	PDA
Hepacic	chronic cholestasis	+	+	+	+	−	+	−	+	+	+	+	−	+
	liver dysfunction	+	+	+	+	+	+	+	+	+	+	+	−	+
	GOT/GPT/TB	262/134/18.4	102/126/1.2	NAV	207/291 /12	90/104/0.8	414/274/17.1	202/281/0.7	85/92/2.1	81/84/0.6	96/107/10.3	321/298/2.5	38/21/0.77	Post-trasplan-tation
	paucity of intrahepatic interlobular bile ducts	+	N.D	−	N.D	+ bilateral hypoplasia	N.D	−	+	N.D	−	+	N.D	
Renal	structural defects	−	−	−	−	−	−	−	−	−		−		
	renal failure	+	−	+	−	+	−	−	−	−	−	−	−	−
Family history		+	−	+	−	−	−	−	−	−	−	−	−	−

+: Present, −: Absent; NAP, Not applicabale; NA, not available; ND, biopsy not performed.
ASD, atrial septal defect; TOF, tetralogy of Fallot; PS, pulmonary stenosis; PD, patient ductus arteriosus.

DHPLC (Escary *et al.* 2000). We have taken all of these factors into account in the design of the assay.

The DHPLC analysis system used in the present study (Transgenomic) allowed us to write a computer script to analyze all the PCR amplicons generated from various portions of the *JAG1* gene in a serial manner at optimized conditions determined individually for each amplicon. This script enabled us to analyze the entire gene overnight in an automated manner. The complete script is available on our Website at http://www.dhplc.jp, both in text and binary format. The binary format script can be directly loaded onto the controlling units of Transgenomic DHPLC systems.

Mutation analysis of patients with Alagille syndrome

The mutations identified in the present study and the associated clinical features are summarized in Table 4. We identified heterozygous *JAG1* mutations in 13 (72%) of the 18 probands enrolled in the study, including five frameshift mutations, three nonsense mutations, two splice-site mutations, and two missense mutations, and one deletion of the 5′ end of the 5′ UTR and the coding region including the start codon. An IVS11+1G>A splicing mutation was detected in two unrelated patients. Eight of the mutations, deletion including start codon, 776G>T, 1026–1027 ins T, IVS11+1G>A, 1720G>C, 2238–2239 ins A, 2650C > T, and 3012–3024 del TTCCCCTTCAGCG have not been previously described. Known SNPs in the *JAG1* gene that are published in the dbSNP database (www.ncbi.nlm.nih.gov/projects/SNP/) were detected, including rs1051415, rs1801138, rs3178014, and rs1051419. No apparent correlation between genotype and phenotype was observed.

Nonsense mutations, as well as deletions and insertions leading to frameshifts, can unambiguously be identified as disease-causing mutations in *JAG1*. The G to A mutation that occurred at the invariant G base of the splice-donor sites flanking exons 11 is most likely pathogenic. This intronic sequence variant was not found in 100 ethnically matched controls. We further attempted to quantify the pathogenicity of these sequence variants by using the NNSplice program (http://www.fruitfly.org/seq_tools/splice.html) (Reese et al. 1997). The NNSplice program calculates the probability of splicing at a specific splicing site. The IVS11+1G>A mutation reduces the probability score from 0.98 to null. These data support the notion that the IVS11+1G>A mutation may affect normal splicing. AU2

Two heterozygous missense substitutions, G259V and V574L, were identified in exon 6 and exon 13, respectively. These missense substitutions are likely to be pathogenic because G259V and V574L residues were conserved in *Macaca mulatta*, *Mus musculus*, *Rattus norvegicus*, and *Danio rerio*, and were not found in 100 ethnically matched controls. We clinically evaluated the parents of the patient with the V574L substitution and confirmed that they showed no signs of Alagille syndrome and the parents were tested for the V574L substitution and found not to have the substitution. Hence, the V574L substitution is likely to have occurred *de novo*. A complete study to support the pathogenicity of the substitution should include a molecular genetic evaluation of the parents to confirm parentage, but the parents declined paternity testing. Because the base substitution 1720G>C leading to V574L occurred at the 3′ end of exon 13, the base substitution might have affected the splicing of intron 13. We attempted to quantify the pathogenicity of the sequence variant using the NNSplice program. The V574L mutation reduced the probability score from 0.94 to 0.5. We could not determine whether *G259V* occurred *de novo*.

In the remaining five patients, all the coding exons of *JAG1* were amplified by PCR and then sequenced in parallel, but no pathogenic mutations were identified. The possibility that the entire *JAG1* gene had been deleted was excluded in four of the five patients because each of the four patients was heterozygous for at least one SNP in the *JAG1* gene. We further screened these five patients without *JAG1* mutations for *NOTCH2* mutations using DHPLC, but no mutations were identified. In these five patients, all the coding exons of *NOTCH2* were amplified using PCR and then sequenced, but no pathogenic mutations were identified.

Known SNPs in the *NOTCH2* gene that are published in the dbSNP database (www.ncbi.nlm.nih.gov/projects/SNP/) were detected, including rs6685892, rs2604045, rs1616532, and

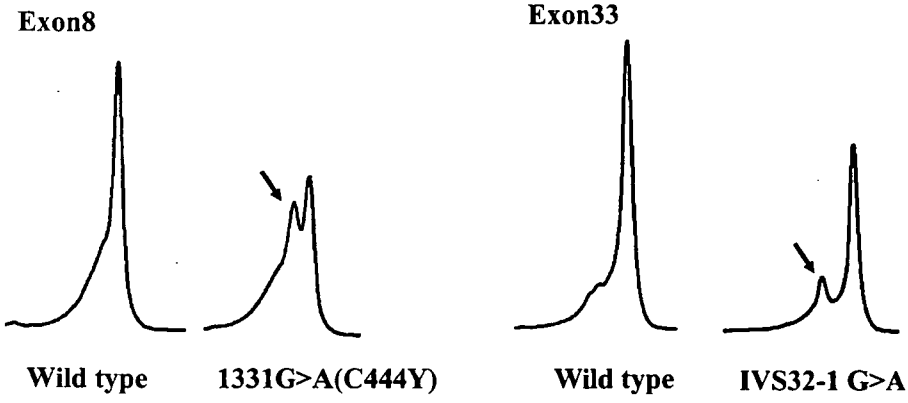

FIG. 3. DHPLC elution profiles of *NOTCH2* mutations previously described (McDaniell *et al.*, 2006). The column temperatures were 61.0°C and 60.0°C for exons 8 and 33, respectively. Only the chromatogram with the best resolution of the heteroduplex peak is shown for each mutation.

rs17024525, 3980A>G, 4014C>T (McDaniell *et al.* 2006) as well as newly identified SNPs including 3779G>A and IVS8-6 G>A. A population survey of 100 ethnically matched controls revealed that four individuals were heterozygous for the 3779G>A substitution. The IVS8-6 G>A substitution was not found in 100 ethnically matched controls but the father of the Alagille syndrome patient with the IVS8-6 G>A substitution showed no signs of Alagille syndrome and found to have the substitution. Hence, the IVS8-6 G>A substitution is most likely a rare variant that does not cause disease. However, given the reduced penetrance of Alagille syndrome, it is possible that the IVS8-6 G>A substitution is disease causing yet the father was unaffected phenotypically.

Site-directed mutagenesis of known NOTCH2 *mutant alleles and their analysis by DHPLC*

PCR amplicons flanking the previously described mutant alleles of the *NOTCH2* gene were generated using the SOE-PCR method, and direct sequencing of the mutagenesis products confirmed that the desired mutant fragments had been successfully generated (data not shown). Equimolar quantities of reference wild-type PCR product and mutant PCR product were mixed, reannealed, and analyzed by DHPLC. Representative DHPLC elution profiles are shown in Figure 3. The chromatograms of the PCR products generated by site-directed mutagenesis exhibited multiple peaks, whereas those of the PCR products amplified from known wild-type homozygotes exhibited a sharp, single peak.

DISCUSSION

In the present study, we developed a DHPLC-based method allowing the entire coding region of the *JAG1* gene and the *NOTCH2* gene to be screened for point mutations, and small deletions and insertions. *JAG1* mutations were identified in more than two-thirds (13 out of 18) of the patients who had been clinically diagnosed as having the Alagille syndrome. In the remaining five patients, all the coding exons of *JAG1* were amplified by PCR and then sequenced in parallel, but no pathogenic mutations were identified. Subsequent analysis of the *NOTCH2* gene in these five patients using both DHPLC screening and complete sequencing of the coding exons failed to identify any mutations. Hence, the negative results for the DHPLC mutation screening for the *NOTCH2* gene were validated. The ability of the DHPLC-based method to detect all previously described *NOTCH2* mutant alleles generated by site-directed mutagenesis supports the notion that the DHPLC protocol described herein is a sensitive method, even if we did not identify any Alagille syndrome patients with *NOTCH2* mutations in the present study.

In 5 of the 18 patients we did not find a micromutation using DHPLC. Several factors may be responsible for a detection rate of less than 100%. First, some patients may have large deletions spanning one or several exons or even the entire *JAG1* and *NOTCH2* locus, which would be undetectable using our methodology. Large deletions can be detected on a DHPLC platform using a recently developed method called multiplex PCR/liquid chromatography assay (Dehainault *et al.* 2004; Udaka *et al.* 2006a, 2006b). A multiplex PCR with unlabeled primers enables simultaneous amplification of multiple exons under semiquantitative conditions and the PCR products separated by DHPLC are quantitated by fluorescence detection. Second, these patients may have mutations deep within introns or in other parts of the *JAG1* and *NOTCH2* gene that were not studied, such as the promoter. Finally, some patients enrolled in the present study may have a different condition that resembles Alagille syndrome such as Byler disease and other hereditary forms of intrahepatic cholestasis (Bull 2002).

The use of the COPPER plate (Kosaki *et al.* 2005; Udaka *et al.* 2005b; Aramaki *et al.* 2006; Udaka *et al.* 2006a) enables all the exons to be simultaneously amplified on a 96-well format PCR plate under the same cycling conditions, while the use of the computer script enables a completely automated DHPLC analysis of all the exons. These two features minimize the labor required by laboratory workers.

The implementation of the screening method for *JAG1* described herein will help medical geneticists confirm their clinical impressions. In the present study, eight patients had undergone liver biopsy before the diagnosis was confirmed by genetic testing. Since liver biopsy is a relatively invasive procedure when compared with genetic testing which is minimally invasive, we suggest that wider availability of the genetic screening test, as exemplified in the present study, would be beneficial to the Alagille patients and their families.

Precise documentation of mutations has clinical ramifications in genetic counseling of Alagille syndrome patients and their families. It is important to note that gonadal mosaicism has been documented in this syndrome (Giannakudis *et al.* 2001). Lack of the mutant allele in the parents' peripheral blood or father's sperm will be informative for the parents in terms of a low recurrence risk (Niu *et al.* 2006). The possibility of prenatal diagnosis in subsequent pregnancies could further reassure the parents.

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Address reprint requests to:
Kenjiro Kosaki, M.D., Ph.D., F.A.C.M.G.
Department of Pedaitrics
Keio University School of Medicine
35 Shinanomachi
Shinjuku-ku, Tokyo 160-8582, Japan

E-mail: kkosaki@sc.itc.keio.ac.jp

ELECTRONIC LETTER

The *Shwachman–Bodian–Diamond syndrome* gene mutations cause a neonatal form of spondylometaphysial dysplasia (SMD) resembling SMD Sedaghatian type

Gen Nishimura, Eiji Nakashima, Yuichiro Hirose, Trevor Cole, Phillip Cox, Daniel H Cohn, David L Rimoin, Ralph S Lachman, Yoshinari Miyamoto, Bronwyn Kerr, Sheila Unger, Hirofumi Ohashi, Andrea Superti-Furga, Shiro Ikegawa

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The *Shwachman–Bodian–Diamond syndrome* (*SBDS*) gene is a causative gene for Shwachman–Diamond syndrome, an autosomal recessive disorder with exocrine pancreatic insufficiency, bone marrow dysfunction and skeletal dysplasia. We report here on two patients with skeletal manifestations at the severest end of the phenotypic spectrum of *SBDS* mutations. An 11-year-old Japanese girl presented with neonatal respiratory failure necessitating lifelong ventilation support, severe short stature and severe developmental delay. She developed neutropenia in infancy, and decreased serum amylase was noted in childhood. A British boy was a stillbirth with pulmonary hypoplasia and hepatic fibrosis found on autopsy. Both cases had neonatal skeletal manifestations that included platyspondyly, lacy iliac crests and severe metaphysial dysplasia, and thus did not fall in the range of the known Shwachman–Diamond syndrome skeletal phenotype but resembled spondylometaphysial dysplasia (SMD) Sedaghatian type. The girl harboured a recurrent mutation (183TA→CT) and a novel missense mutation (79T→C), whereas the boy carried two recurrent mutations (183TA→CT and 258+2T→C). We also examined *SBDS* in one typical case with SMD Sedaghatian type and eight additional cases with neonatal SMD, but failed to discover *SBDS* mutations. Our experience expands the phenotypic spectrum of *SBDS* mutations, which, at its severest end, results in severe neonatal SMD.

The *Shwachman–Bodian–Diamond syndrome* (*SBDS*) gene (GeneBank AC079920) is a causative gene for Shwachman–Diamond syndrome (SDS; OMIM 260400), an autosomal recessive disorder with exocrine pancreatic insufficiency, haematological abnormalities and generalised skeletal dysplasia.[1 2] Most *SBDS* mutations are caused by gene conversion between *SBDS* and its neighbouring pseudogene (*SBDSP*; GeneBank AC005236). The SBDS protein, which does not have sequence homology to known functional domains, is assumed to have a role in RNA metabolism.[3–4] This presumption may account for pleiotropic effects of *SBDS* mutations and multi-system involvement in SDS.

The clinical phenotype of SDS is diverse.[5–7] Pancreatic insufficiency commonly presents with steatorrhoea in childhood, but it may develop first in the older age. Haematological abnormalities range from cyclic or persistent neutropenia through pancytopenia with a specific risk of evolution into myelodysplastic syndrome to acute myeloid leukaemia.[8 9] Hepatic, renal and neurological dysfunctions are rare associations of SDS. SDS manifests radiologically as generalised metaphysial dysplasia, which causes only mild to moderate short stature in general. A small subset of affected individuals may present with respiratory distress due to a narrow thorax with short ribs, resembling Jeune syndrome (OMIM 208500), but the disorder is usually not lethal.[10]

We report here on two compound heterozygotes for *SBDS* mutations whose manifestations can be regarded as the severest end of the phenotypic spectrum known for mutations in the gene. One patient has survived respiratory failure with lifelong ventilation support, and showed severe short stature and developmental delay. The other was a stillbirth with pulmonary hypoplasia. Their extraskeletal manifestations may fit a diagnosis of SDS. However, their skeletal phenotype was not typical of SDS, but resembled a mild form of another skeletal dysplasia termed spondylometaphysial dysplasia (SMD) Sedaghatian type (OMIM 250220).[11]

MATERIALS, METHODS AND RESULTS

Clinical reports

Patient 1

The girl was born to a healthy, non-consanguineous Japanese couple. She was delivered by caesarean section at 38 weeks gestation because of breech presentation. Her birth length was 40 cm (−4.9 SD), weight 1870 g (−3.1 SD) and head circumference 32.7 cm (−0.3 SD). Apgar scores at 1/5 min were 7/9. She had a narrow thorax and rhizomelic shortening of the limbs. Her profound hypotonia with poor breathing prompted assisted ventilation, which was enduringly continued. Laryngoscopy showed tracheomalacia with stenosis at 1 month of age. At 4 months of age, she developed intermittent neutropenia, which required granulocyte colony-stimulating factor therapy that has been intermittently continued thereafter. At 30 months of age she presented with seizures, which were responsive to epilepsy medicines. Decreased serum isoamylase (4 IU/l) attracted attention at 5 years of age, but no overt symptoms of malabsorption were noted. She is currently 11 years old and is markedly retarded, along with severe hypotonia and bilateral hearing impairment (no reaction for 90 dB). Her height is 93 cm (−7.5 SD). A skeletal survey at birth showed a narrow thorax, short ribs with cupped anterior ends, severe dense platyspondyly, lacy iliac crests, delayed ossification of the caudal ilia, and metaphysial cupping and irregularity of the tubular bones (fig 1). A tentative diagnosis of SMD Sedaghatian type was made at that time. The skeletal alterations at 5 and 11 years of age comprised thoracic narrowing, posteriorly wedged vertebral bodies without platyspondyly, iliac hypoplasia, metaphysial irregularity with epiphysial plate widening and large epiphyses of the long bones,

Abbreviations: SBDS, Shwachman–Bodian–Diamond syndrome; SDS, Shwachman–Diamond syndrome; SMD, spondylometaphysial dysplasia