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Molecular Analysis of 24 Alagille Syndrome Families Identifies a Single Submicroscopic Deletion and Further Localizes the Alagille Region within 20p12

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Summary

Alagille syndrome (AGS) is a clinically defined disorder characterized by cholestatic liver disease with bile duct paucity, peculiar facies, structural heart defects, vertebral anomalies, and ocular abnormalities. Multiple patients with various cytogenetic abnormalities involving 20p12 have been identified, allowing the assignment of AGS to this region. The presence of interstitial deletions of varying size led to the hypothesis that AGS is a contiguous gene deletion syndrome. This molecular analysis of cytogenetically normal AGS patients was performed in order to test this hypothesis and to refine the localization of the known AGS region. Investigation of inheritance of simple tandem repeat polymorphism alleles in 67 members of 24 cytogenetically normal Alagille families led to the identification of a single submicroscopic deletion. The deletion included loci D20S61, D20S41, D20S186, and D20S188 and presumably intervening uninformative loci D20S189 and D20S27. The six deleted loci are contained in a single YAC of 1.9 Mb. The additional finding of multiple unrelated probands who are heterozygous at each locus demonstrates that microdeletions at known loci within the AGS region are rare in cytogenetically normal patients with this disorder. This suggests that the majority of cases of AGS may be the result of a single gene defect rather than a contiguous gene deletion syndrome.

Introduction

Alagille syndrome (AGS) is a clinically defined disorder characterized by cholestatic liver disease with intrahepatic bile duct paucity, peculiar facies, structural heart defects, vertebral anomalies, and ocular abnormalities (Alagille et al. 1975, 1987; Deprettere et al. 1987; Mueller 1987). Additional clinical features may include growth retardation, long bone abnormalities, and renal disease (Alagille et al. 1975, 1987; Deprettere et al. 1987). There is a wide range of clinical expression among family members within each family and in each independent organ system within each individual (Schulman et al. 1984).

AGS was first fully described in 1975, and pedigree analysis suggested that its inheritance was autosomal dominant (Alagille et al. 1975, 1987). Segregation analysis of AGS in 33 families has confirmed autosomal dominant transmission with nearly complete penetrance (Dhorne-Pollet et al. 1994). The association between AGS and chromosome 20p was first noted in a case report describing a patient with del(20)p11.23-pter and a phenotype consistent with AGS (Byrne et al. 1986). This large deletion included almost the entire short arm of chromosome 20 and was therefore easily visible by cytogenetic analysis. Since that time, a total of nine Alagille probands with various del(20p) have been described; the incidence of these deletions appears to be $\leq 5\%$ (Byrne et al. 1986; Schnittger et al. 1989; Anad et al. 1990; Legius et al. 1990; Zhang et al. 1990; Teebi et al. 1992). In one report, del(20p) cosegregated with AGS in a mother and infant (Anad et al. 1990). The size of the deletions seen in the reported cases has varied between families, and the overlap of the deletions allowed the definition of a smaller Alagille region at 20p11.23-p12.2 (Anad et al. 1990). In addition, a translocation, t(2;20)(q21.3;p12), that segregates with AGS in a two generation family has recently been reported (Spinner et al. 1994). Molecular analysis of a somatic cell hybrid containing the isolated der(20) has localized the translocation breakpoint distal to D20S61 and D20S56 yet proximal to D20S115 within band 20p12 (Spinner et al. 1994). Linkage analysis performed on a three-generation family with AGS has confirmed localization to chromosome 20p in a cytogenetically normal family (Hol et al. 1995).

Despite the increasing availability of highly heterozygous chromosome 20 microsatellite markers, there are no studies examining multiple loci in a large series of cytogenetically normal Alagille patients. The current study of inheritance of simple tandem repeat polymor-

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phisms (STRPs) in cytogenetically normal AGS families was undertaken to determine the frequency of submicroscopic deletions and to identify any deleted and flanking loci.

Subjects and Methods

Recruitment of Subjects and Preparation of Samples

Probands with AGS were identified through the Pediatric Gastroenterology clinics at the Children's Hospital of Philadelphia (Philadelphia) and the Wyler Children's Hospital (Chicago) as well as through referrals from geneticists and pediatric gastroenterologists at national meetings or resulting from publications. The diagnosis of AGS was confirmed by review of clinical data by at least two pediatric gastroenterologists. Probands all presented with liver disease, and the diagnosis was made by characteristic liver biopsy findings with the presence of at least two other major criteria of AGS (excluding facies). Alagille patients were enrolled under institutional review board–approved protocols at either Children's Hospital of Philadelphia or the Wyler Children's Hospital. High-resolution cytogenetic analysis was performed as part of a cytogenetic survey of AGS (N. B. Spinner, data unpublished). Twenty-one patients with normal findings were then further studied by STRP analysis. Cytogenetic analysis was not available for three probands who were included in this study. Genomic DNA was extracted from whole blood or Epstein Barr virus–transformed cell lines by standard methods.

STRP Analysis

Primers designed for use in PCR amplification of each STRP locus were obtained commercially (Research Genetics MapPairs) or were chosen from sequences available in the Genome Database and synthesized in the University of Pennsylvania Howard Hughes Medical Institute oligonucleotide core facility. PCR reactions were performed using standard components with 50–150 ng of genomic template DNA. Twenty percent of the total forward primer used in the PCR mixture was labeled using ^{32}P-γ-dATP via a standard kinasing reaction (Sambrook et al. (1989). Cycling conditions in each case started with an initial 4-min denaturation at 92°C followed by 45 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Final PCR products were resolved by electrophoresis over a 5% acrylamide/urea gel and visualized by autoradiography.

YAC Analysis

YACs were identified via computer searches of the Human Genome Database for specific loci (e-mail address: http://www-genome.wi.mit.edu). YAC colonies were obtained from the CEPH/Généthon libraries via the Human Genome Center for Chromosome 22. YACs were grown in liquid culture, and total DNA extracts were prepared by standard protocol (Green and Olson 1990). Radiolabeled PCR products were generated as above, with annealing temperature ranging from 60°C to 63°C. Reactions were performed without radiolabeled primer for some loci (i.e., D20S177), and these products were resolved by electrophoresis over an ethidium bromide–stained 2.5% agarose gel. The annealing temperature for these reactions was 57°C.

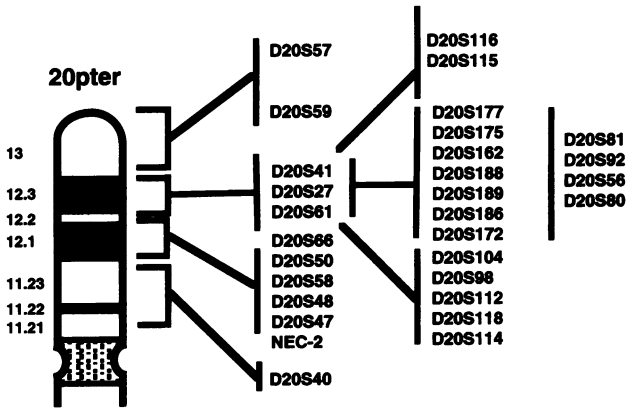

Figure 1 Chromosome 20p with relative positions of STRP loci used in this study. Positions of STRP loci were estimated from Genome Database map information and references (NIH/CEPH Collaborative Mapping Group 1992; Ohagi et al. 1992; Weissenbach et al. 1992; Melis et al. 1993).

Results

In total, 67 individuals who were members of 24 unrelated Alagille families were studied with 30 different STRP loci. The loci were selected for proximity to the Alagille region and also for heterozygosity; their relative positions are shown in figure 1. All loci were initially analyzed for every available family; however, as finer mapping information regarding relative position became available, some were eliminated from further study. The number of loci analyzed per family ranged from 8 to 20. The high number of alleles and increased heterozygosity of the STRPs makes informative matings more likely than with RFLPs and improves the efficiency of deletion screening (Weber 1990). Deletions are identified when a proband fails to inherit an allele from a parent at a particular locus and is therefore hemizygous at that locus. The genotype of each proband at 12 selected loci is shown in table 1. A single submicroscopic deletion was detected in patient 1, discussed further below. In several cases (indicated on table 1), paternal DNA samples were not available, thereby reducing the informativeness of the studies at homozygous loci in those individuals.

A submicroscopic deletion was identified in 1 family of the 24 studied (patient 1). This particular child has no clinical features to distinguish her from other patients

Table 1

Genotype of Alagille Probands at Various 20p Loci

Patient No.	D20S98	D20S112	D20S104	D20S172	D20S61	D20S41	D20S186	D20S189	D20S27	D20S188	D20S162	D20S175
1	H	H	H	H	D	D	D	U	U	D	H	H
2	H	H	H	H	U	H	H	U	H	H	H	H
3[a]	H	H	U	H	H	U	U	U	U	U	H	H
4[b]	H	H	H	U	H	H	H	U	H	U	H	U
5	...	...	H	U	H	...	H	H	U	U	H	U
6[b]	H	H	...	H	H	H	H	U	H	...	H	...
7[a]	...	H	H	H	H	U	U	H	H	U	H	H
8[a]	H	H	H	H	U	H	H	U	U	H	H	H
9	H	...	H	U	H	H	U	U	U	...	H	H
10[b]	...	H	H	H	H	H	H	U	U	H	U	U
11	...	...	H	U	U	H	H	U	U	H	H	H
12	...	...	U	U	H	H	H	U	U	H	H	H
13	...	...	U	U	U	H	H	H	U	H	H	U
14	...	...	H	H	H	...	H	H	H	H	U	H
15	...	...	H	H	U	H	H	U	H	H	H	H
16	...	...	H	H	H	...	H	H	H	U	H	U
17	...	...	H	U	H	H	H	H	U	...	H	H
18	...	...	H	H	H	...	H	U	H	...	H	...
19	...	...	H	H	H	H	H	H	U	H	H	H
20	...	...	H	U	H	H	H	U	U	H	H	H
21[b]	...	...	H	U	H	...	H	H	U	U	H	H
22	...	...	H	U	H	U	U	U	U	U	H	U
23	...	...	H	H	H	H	H	H	H	H	H	H
24	...	...	U	U	H	H	U	U	H	H	U	H

NOTE.—"H" indicates that the proband is heterozygous at the locus indicated; "U" indicates that the mating is uninformative. The mothers of patients 2, 9, 21, and 24 are hypothesized to be affected, on the basis of physical findings, and, for 21 and 24, the presence of affected siblings.

[a] Not examined cytogenetically.

[b] Samples from father of proband were not available.

with AGS. She presented with jaundice and elevated serum transaminases at 6 wk of age, at which time histologic examination of a percutaneous liver biopsy was notable for characteristic changes of cholestasis and ductal paucity. A cardiac murmur was noted, and an echocardiogram revealed stenosis of the pulmonary artery. Spinal films demonstrated several butterfly vertebrae, and subsequent ocular examination was positive for the presence of posterior embryotoxon. This patient is now 5 years old and has normal neurologic development. Xanthomata and hypercholesterolemia which developed by 30 mo of age have almost completely resolved following a surgical partial external biliary diversion (Whitington and Whitington 1988).

Cytogenetic studies of this patient revealed an apparently normal karyotype at the 550–600-band level (data not shown). The proband fails to inherit a maternal allele at D20S161, D20S41, D20S186, and D20S188. Two intervening loci, D20S189 and D20S27, were uninformative but are assumed to be within the deletion. Figure 2 shows the STRP alleles for each individual in this family at each of the deleted loci and a representative uninformative locus. The patient is heterozygous at loci flanking this region (D20S172 and D20S162), which demonstrates that the deletion does not extend significantly beyond the loci shown to be hemizygous. Because this child is heterozygous at 12 of 14 additional loci with inheritance of maternal alleles in each case, nonmaternity is not a reasonable explanation for the findings. The mother is heterozygous at D20S61, D20S189, and D20S27; therefore the deletion causing AGS is a new mutation in this child.

YACs associated with STRP loci within or near the deletion have been identified through computer searches of the first generation physical map of the human genome (Cohen et al. 1993). The database reveals a single YAC of 1.9 Mb (address 940D11), which contains STRP loci D20S61, D20S41, D20S189, and D20S188. Genomic DNA was extracted from YAC cultures grown from 940D11 obtained from the CEPH mega-YAC library. YAC DNA was analyzed for the presence of deleted and flanking STRP loci. The presence of D20S61, D20S41, D20S189, and D20S188 reported in the Genome Database was confirmed by PCR using YAC 940D11 as a template (data not shown). In addition, figure 3 demonstrates the localization of loci D20S27 and D20S186 to YAC 940D11. Distal locus D20S177 was absent from YAC 940D11. Proximal flanking loci D20S104 and

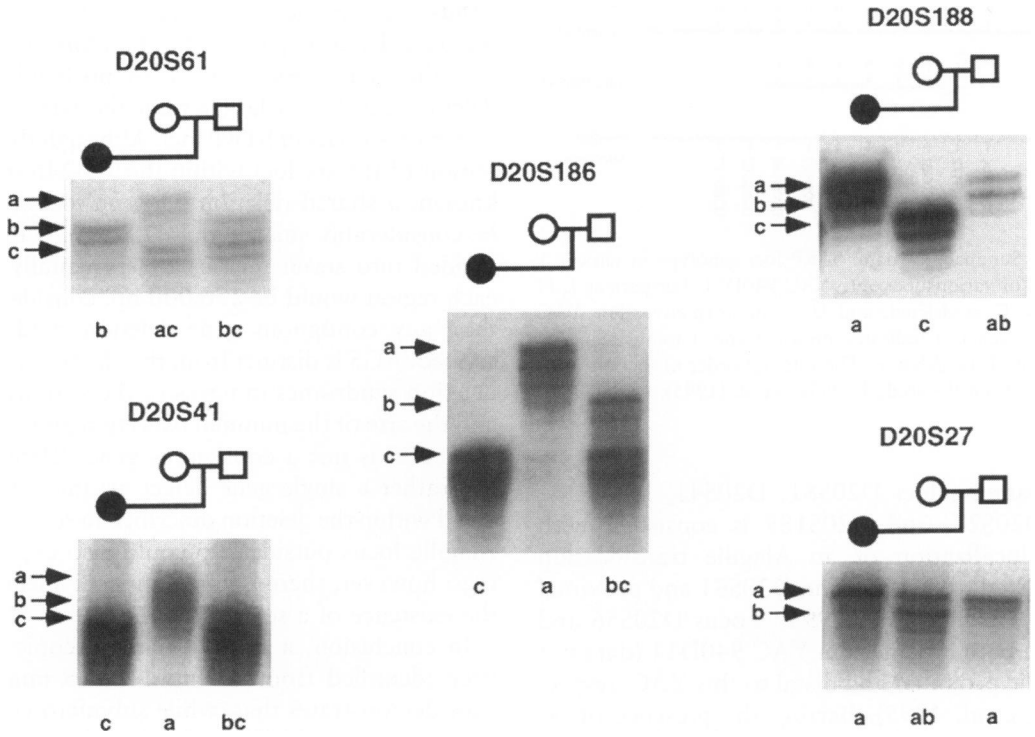

Figure 2 STRP analysis results at various STRP loci. Failure to inherit a maternal allele is demonstrated at D20S61, D20S41, D20S188, and D20S186. D20S27 is uninformative.

D20S172 were also absent from 940D11, as was distal flanking locus D20S162 (data not shown). The presence of all six deleted loci on a single nonchimeric YAC of 1.9 Mb demonstrates their physical proximity. Figure 4 summarizes the genotype of the deletion patient at each locus as well as the presence or absence of each locus on YAC 940D11. Proximally, loci D20S104 and D20S172 (heterozygous in patient 1) were not detected in YAC 940D11 DNA, so the proximal end of the YAC falls between D20S186 and these markers, as does the proximal deletion breakpoint. Similarly, the distal deletion breakpoint and YAC end fall between D20S188 and D20S162. The breakpoints of the deletion therefore may or may not be included within YAC 940D11. The region

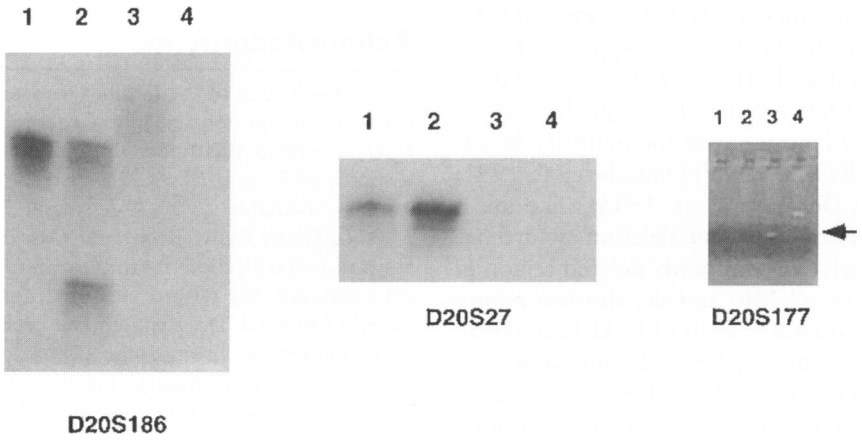

Figure 3 STRP analysis for YAC 940D11 at loci D20S186, D20S27, and D20S177. D20S186: Lane 1, YAC 940D11; Lane 2, Human genomic DNA; Lane 3, YAC 881H2; and Lane 4, Water. D20S27, same as D20S186 except Lane 3, YAC 801E6. D20S177: Lane 1, Water; Lane 2, 940D11; Lane 3, YAC 881H2; Lane 4 = Water. For D20S177, the arrowhead indicates the expected band. Nonspecific amplification of additional bands is evident in the human genomic lane. YAC 881H2 is mapped distal to YAC 940D11 on chromosome 20p, and YAC 801E6 is mapped to chromosome 22 (Genome Database data).

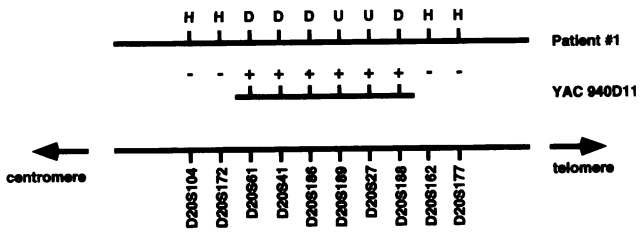

Figure 4 Summary of data: STRP loci genotype in patient 1 and localization of various loci onto YAC 940D11. For patient 1, H = heterozygous, D = deleted, and U = uninformative. For YAC 940D11, a plus sign (+) indicates presence and a minus sign (−) indicates absence of the deletion. The internal order of loci on YAC 940D11 is taken from the study by Pollet et al. (1995).

identified spanning loci D20S61, D20S41, D20S186, D20S189, D20S27, and D20S188 is consistent with our earlier localization of an Alagille translocation breakpoint distal to D20S56 and D20S61 and proximal to D20S115 (Spinner et al. 1994). Locus D20S56 and D20S115 are both absent from YAC 940D11 (data not shown) and lie proximal and distal to this YAC, respectively (Pollet et al. 1995). Barring the presence of rearrangements within the YAC, yet preserving the STRP loci, this narrows the Alagille critical region to the 1.9-Mb genomic segment contained in YAC 940D11 and possibly a small amount of flanking DNA.

Discussion

The finding of a submicroscopic deletion in a patient with AGS confirms the localization of the AGS region to a small segment of chromosome 20. Furthermore, the absence of deletions in 23 patients is also of importance in understanding the genetic basis of this syndrome. It has been speculated that AGS falls into the category of contiguous gene deletion syndromes. In these disorders, a finite but variable number of clustered genes are deleted, causing a heterogeneous phenotype (Emanuel 1988). Molecular studies of other contiguous gene deletion syndromes (i.e., Prader-Willi, DiGeorge) have demonstrated deletions to be present in the majority of patients studied (Nicholls et al. 1989; Hamabe et al. 1991; Driscoll et al. 1992; Desmaze et al. 1993). The exact sizes of the known contiguous gene deletion syndromes are unknown; however, the commonly deleted region in DiGeorge syndrome is ~2 Mb, and the shortest region of overlap in these patients is ≥500 kb (Driscoll et al. 1992). In the present study of AGS, only one submicroscopic deletion could be detected in 24 patients studied. Although the observed deletion is certainly large enough to postulate a contiguous gene deletion in patient 1, there is no evidence to suggest that a smaller deletion is shared in every affected individual. Examination of the data in table 1 demonstrates the low likelihood of failure to identify overlapping deletions by this technique. Probands 2–24 are heterozygous at multiple loci within the region deleted in patient 1. Therefore, if a deletion is hypothesized to exist in all 24 probands, the shared deletion can be no larger than the largest interval between two adjacent STRP loci. Although the exact distribution of the six loci within the 1.9-Mb interval is unknown, a shared deletion between loci would have to be considerably smaller than 1.9 Mb. If the YAC were divided into seven regions by six equally spaced loci, each region would be 270,000 bp, considerably smaller than any contiguous gene deletion syndrome yet described. AGS is distinct from the classic contiguous gene deletion syndromes in terms of the scarcity of deletions and the size of the minimal overlap region. This suggests that AGS is not a contiguous gene deletion syndrome but rather a single gene defect arising from a gene located within the deletion described here. An independent Alagille locus outside 20p could also explain our findings; however, there is currently no evidence to support the existence of a second locus.

In conclusion, a single submicroscopic deletion has been identified from among 24 AGS families studied. This demonstrates that, while submicroscopic deletions occur in cytogenetically normal AGS patients, such cases are rare. The deletion described in this study clearly includes four 20p loci, D20S61, D20S41, D20S186, and D20S188 and presumably the intervening ambiguous loci D20S189 and D20S27. Each of these loci localize to a single YAC of 1.9 Mb within the smallest chromosome 20 deletion described in AGS. The scarcity of microdeletions in cytogenetically normal AGS patients would not be expected on the basis of comparisons to other contiguous gene syndromes. Although AGS is caused by deletions of various sizes in some instances, deletions in this region cannot explain the majority of cases, which may be caused by single gene defect(s) within the AGS region.

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