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Genetics

A human vascular disorder, supravalvular aortic stenosis, maps to chromosome 7

(linkage analysis/elastin/Williams syndrome)

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ABSTRACT **The pathogenesis of vascular disease is unclear, but genetic factors play an important role. In this study we performed linkage analyses in two families with supravalvular aortic stenosis, an inherited vascular disorder that causes narrowing of major arteries and may lead to cardiac overload and failure. DNA markers on the long arm of chromosome 7 (*D7S371*, *D7S395*, *D7S448*, and *ELN*) were linked to supravalvular aortic stenosis in both families with a combined logarithm of likelihood for linkage (lod score) of 5.9 at the *ELN* locus. These findings indicate that a gene for supravalvular aortic stenosis is located in the same chromosomal subunit as elastin, which becomes a candidate for the disease gene.**

Supravalvular aortic stenosis (SVAS) is an inherited vascular disorder (1). As its name implies, narrowing of the ascending aorta is a dominant feature of this disease, but other arteries, including the pulmonary arteries, may be affected. When uncorrected, SVAS may lead to increased intracardiac pressure, myocardial hypertrophy, heart failure, and death.

The incidence of SVAS is estimated to be 1 in 25,000 live births (personal communication, Birth Defects and Genetic Diseases Branch Personnel, Metropolitan Atlanta Congenital Defects Program, Centers for Disease Control and Prevention, Atlanta). The vascular abnormalities typical of SVAS can be inherited as an isolated, autosomal dominant trait (1–3) or as part of a second disease, Williams syndrome, a developmental disorder that affects multiple organ systems (2–4). In addition to vascular disease, manifestations of Williams syndrome include learning disability, an unusually gregarious personality, premature greying of the hair, premature aging of the skin, joint laxity early in life followed by joint contractures, dysmorphic facial features and infantile hypercalcemia. The relationship between SVAS and Williams syndrome is undefined.

Diagnosis of SVAS has been based on family history, physical examination, and echocardiography. Unfortunately, these tests may be ambiguous, making early detection of this disorder difficult. The recent advent of high-quality two-dimensional and color Doppler-flow echocardiography have improved noninvasive screening for SVAS (5), but invasive tests such as cardiac catheterization and angiography are more sensitive. Currently vascular surgery is the only treatment option for SVAS.

Although SVAS was first described in 1842 (6), the metabolic basis of this disorder is unknown. Pathological studies demonstrated that hypertrophy and hyperplasia of vascular smooth muscle were important features of this disease, but the molecular mechanisms underlying these findings were obscure. To improve our understanding of SVAS, we conducted linkage analyses using a large family, with 13 affected individuals, and a smaller family of 4 affected individuals. We found that the SVAS phenotype was closely linked to *D7S371*, *D7S395*, *D7S448*, and *ELN*, DNA markers mapping to the long arm of chromosome 7.

MATERIALS AND METHODS

Phenotypic Evaluation. Kindred 1773 has been described (7, 8). Informed consent was obtained from all study participants in accordance with standards established by local institutional review boards. To determine whether family members and spouses had signs of SVAS or Williams syndrome, physical examinations were performed by a medical geneticist (C.A.M. or C.M.), as described (5).

To determine the phenotype of individuals, all Doppler echocardiographic data were reviewed independently by two pediatric cardiologists (G.J.E. or J.L.). Individuals were classified as affected, uncertain, and unaffected based on catheterization, angiography, and surgical findings (17 individuals, Table 1). If catheterization data were unavailable, phenotype was determined based on echocardiographic impression of narrowing of the aorta at the sinotubular junction and the supravalvular pulmonary region, increased Doppler blood-flow velocity in the ascending aorta, main pulmonary artery, and/or peripheral pulmonary arteries (5). Family members were scored on a scale from −6 (no evidence of SVAS) to +6 (strong evidence of SVAS). For linkage analysis, individuals with impression scores of −2 and lower were classified as unaffected, individuals with scores of +2 and greater were classified as affected, and individuals with scores of −1, 0, and +1 were classified as uncertain. Phenotypic criteria were identical for females and males.

DNA Analysis. Human genomic DNA was purified from leukocytes and lymphoblastoid cell lines (10, 11). Five micrograms of DNA was digested with restriction endonucleases (Molecular Biology Resources, Milwaukee, WI). DNA fragments were separated by agarose gel electrophoresis and transferred to nylon membranes (ref. 12, Hybond-N^+, Amersham). Membranes were hybridized in solution containing 10% polyethylene glycol, 7% SDS, 1.5× standard saline phosphate/EDTA (SSPE; 1× SSPE is 0.18 NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), human placental DNA at 250 μg/ml at 65°C for 24 hr. Plasmids were denatured and labeled with [^{32}P]dCTP (New England Nuclear) by random-primer synthesis (13) to high specific activity (typically 1–5 × 10^9 cpm/μg of DNA). Radiolabeled probe DNAs were hybridized overnight to the human DNA transfers at 65°C in

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Abbreviations: SVAS, supravalvular aortic stenosis; ELN, elastin; lod score, logarithm of likelihood for linkage.

Table 1. Phenotype evaluations of SVAS families

Kindred	Age	V_a, m/s	V_p, m/s	STJ, cm	Score*	Cath	Affected status†
K1773							
I-1	—	—	—	—	—	—	U
I-2	—	—	—	—	—	—	U
II-1	—	—	—	—	—	—	U
II-4	80	—	—	—	—	—	U
II-5	70	1.2	0.9	2.3	−6	—	N
II-6	66	0.9	0.8	2.2	−6	—	N
II-7	68	1.3	1.1	1.9	−2	—	N
II-9	64	2.6	—	1.9	−2	−:AVS	U
II-11	61	1.6	1.4	1.6	0	—	U
II-13	—	—	—	—	—	+	A
III-1	—	—	—	—	—	+:SP	A
III-3	43	1.3	na	—	−3	—	N
III-4	41	1.2	0.8	2.5	−4	—	N
III-7	38	1.0	1.1	1.8	−2	—	N
III-8	37	—	—	—	—	+:SP	A
III-9	33	1.3	0.7	2.4	−6	—	N
III-11	32	1.5	1.2	2.8	−3	+	N
III-13	29	1.5	1.0	1.8	+6	+:SVPS	A
III-14	56	0.8	0.9	2.2	−4	—	N
III-15	52	1.8	0.9	1.8	+3	—	A
III-17	47	—	—	—	—	+:SP	A
III-19	41	—	—	—	—	—	U
III-21	36	1.5	0.9	2.3	−4	—	N
III-22	27	1.4	0.9	2.0	−5	—	N
III-23	32	1.4	0.8	2.8	−5	—	N
III-24	30	2.0	0.6	1.9	+3	+	A
III-26	40	—	—	1.1	+6	+:SP	A
III-28	33	1.6	0.8	2.1	−4	—	N
III-29	30	2.0	—	1.6	−1	−:BAV	U
III-30	37	—	—	—	—	+	N
III-31	36	—	—	—	—	+	N
III-32	34	—	—	—	—	—	U
III-33	31	—	—	—	—	+	A
III-34	29	—	—	—	—	+:SP	A
IV-1	25	1.4	1.0	1.8	−2	—	N
IV-2	23	1.8	0.7	2.6	+2	−:BAV	U
IV-3	12	1.3	0.8	1.7	−3	—	N
IV-4	18	1.6	1.1	2.4	+1	—	U
IV-5	14	1.0	1.0	2.0	−6	—	N
IV-6	16	1.8	1.1	1.9	+1	—	U
IV-7	2	1.0	1.0	1.4	+1	—	U
IV-8	9	2.5	0.9	1.3	+5	—	A
IV-9	7	1.1	0.7	1.8	−4	—	N
IV-10	—	—	—	—	—	+	A
IV-11	21	1.2	1.0	2.8	−5	—	N
IV-12	18	1.4	1.0	1.9	+2	+	A
IV-13	3	3.3	2.2	1.0	+6	—	A
IV-14	11	1.4	0.8	1.7	−6	—	N
IV-15	10	1.1	1.0	1.7	−3	—	N
IV-16	4 mo	1.7	1.7	0.7	+4	—	A
K1779							
I-1	65	—	—	—	+2	—	A
II-1	41	—	—	—	—	+	A
II-3	37	—	—	—	—	—	U
II-4	33	—	—	—	—	+:SP	A
II-5	28	—	—	—	—	—	U
III-1	16	—	—	—	—	+:SP	A
III-2	12	1.4	0.9	2.5	−4	—	N

Table incorporates the phenotypic data for SVAS family members. Doppler-flow studies measured maximum aortic velocity (V_a) in m/s. Normal values for adults (age 15 and older) are 1.0–1.7 m/s, and normal values for children (age 14 and younger) are 1.2–1.8 m/s. Doppler-flow studies also measured maximum pulmonary velocity (V_p) in m/s. Normal values for adults were 0.6–0.9 m/s, and normal values for children were 0.7–1.1 m/s (9). SVAS and supravalvular pulmonic stenosis (SVPS) were also detected in 17 individuals by cardiac catheterization (Cath) or surgical pathology (SP). Impression scores (score) were based on Doppler and two-dimensional echocardiographic findings, including measurement of the sinotubular junction (STJ). na, not applicable.

*Impression scores of +2 and above were considered affected; scores of −2 and below were considered unaffected. Impression scores of −1, 0, and +1 were considered uncertain, unless catheterization or surgical data indicated otherwise.

†A, affected; N, unaffected; U, uncertain. No phenotypic data were available for individuals II-4 and III-32 (K1773) or II-3 and II-5 (K1779), so they were classified as U. Individual II-9 (K1773) was classified as U because of aortic valvular stenosis (AVS). Individuals III-29 and IV-2 (K1773) were classified as U due to bicuspid aortic valves (BAV).

fresh hybridization solution. Membranes were then washed twice for 15 min at room temperature in 0.1× standard saline citrate/0.1% SDS and then washed for 30 min at 65°C. The following probes, known to show restriction fragment length polymorphisms in human genomic DNA, were used: D7S371 (14), D7S448 (15), D7S395 (16), and D7S8 (16).

Polymorphic sequences at the elastin (ELN) locus (17) were amplified by PCR with a final vol of 25 μl containing 200 ng of genomic DNA template. Reactions contained 0.4 μM of each unlabeled oligonucleotide primer (HEIG15: 5′-CGCTCTAGACAAGGCCTGGGGGAAATTTACATCC-3′ and HEIG16: 5′-CGCAAGCTTCTGGAGGCCTGGGAGC-CAGTTTG-3′, ref. 17), 200 μM each of dNTPs (Pharmacia), 1× PCR buffer (10 mM Tris, pH 8.3/50 mM KCl/1.5 mM MgCl), and 1.25 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). Samples were overlaid with mineral oil and processed through 30 PCR cycles: 1.5 min at 94°C, 1 min at 65°C, 1 min at 72°C, and a final extension step of 7 min at 72°C. Products were incubated with *Bst*NI, run on a 4% NuSieve/0.5% LE agarose gel, and stained with ethidium bromide.

Linkage Analysis. Linkage analyses were performed by using the programs MLINK and LINKMAP (18). The logarithms of likelihoods for linkages (lod scores) were calculated at various recombination fractions for each probe. Based on segregation analysis, we assumed an autosomal dominant inheritance of a single gene with a penetrance of ≈0.90. Allele frequencies for markers were from previous calculations (14–17). Male and female recombination fractions were assumed to be equal.

RESULTS

Phenotypic Analyses. We studied two multigenerational families with SVAS (Fig. 1 *A* and *B*). Kindred 1773 was of Irish descent and included 47 family members at risk for SVAS. The second family, K1779, was of German descent and had seven family members at risk for this disorder. Seven affected members of these kindreds required surgical correction of SVAS and at least three died of this disorder; two individuals died in early childhood (18 mo and 3 yr) during catheterization and surgery, respectively, and one died at age 39 of heart failure after refusing surgery. There was no evidence that these families were related.

The clinical features, including variable expression, of affected family members were typical of familial SVAS with one exception. In addition to severe SVAS, one affected member of K1779 (III-1) had learning disability (intelligence quotient of 76), gregarious personality, hoarse voice, joint contractures, and mild dysmorphic facial features. These characteristics satisfied the diagnostic index for Williams syndrome (19).

Segregation analyses indicated an autosomal dominant pattern of SVAS gene inheritance with incomplete pene-

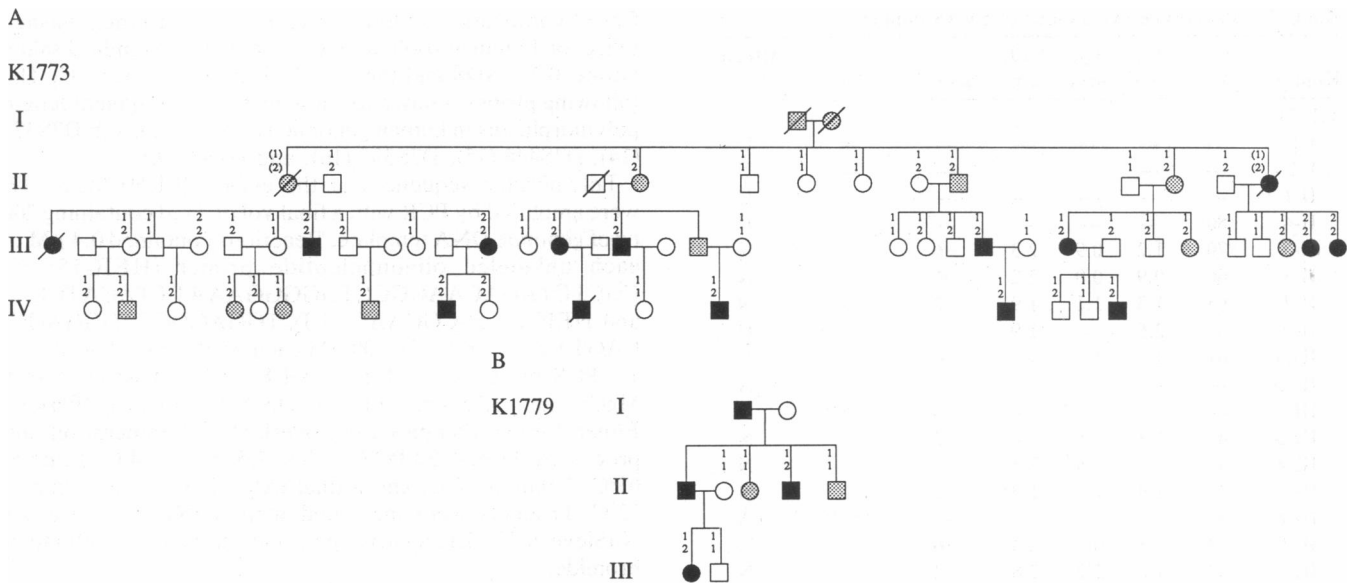

FIG. 1. Pedigree structure and *ELN* genotypes for SVAS families K1773 (*A*) and K1779 (*B*). Affected individuals having the characteristic pattern of elevated Doppler velocity and narrowing of the ascending aorta or pulmonary arteries on echocardiogram are represented by ● (females) and ■ (males). Unaffected individuals are represented by □ or ○. Family members who had an equivocal phenotype or for whom no phenotypic data were available are represented by ⊡ and ⊙. Above each symbol, individual alleles (1 or 2) are listed for the ELN polymorphic PCR marker (17). At this marker locus the restriction enzyme *Bst*NI revealed two alleles within the families. The disease gene cosegregated with the 244-bp allele (allele 2) in both families. Alleles shown in parentheses were inferred. The pedigree structures were altered to protect confidentiality.

trance (data not shown). These data suggested that some SVAS gene carriers appeared unaffected by the disease. To avoid misclassifying individuals, we took a conservative approach to phenotypic assignment. Each individual was given an impression score based on the extent of observed SVAS, supravalvular pulmonic stenosis, or peripheral pulmonary artery stenosis. We used impression scores, coupled with catheterization, angiographic, and surgical data, to classify family members as affected, unaffected, or uncertain. We examined 47 individuals from K1773 and 7 individuals from K1779 (Table 1). As a result, 17 family members were classified as affected, 23 were classified as unaffected, and 14 were classified as uncertain. As history and physical examination for spouses were normal in all but one instance, we assumed that spouses were not affected by this rare disorder; one spouse had a click-murmur, and echocardiogram confirmed mitral-valve prolapse.

Linkage Analysis. To determine the chromosomal location of a SVAS gene, we performed linkage analysis with highly polymorphic DNA markers that span the genome. We also included DNA markers mapping near genes that were candidates for the disease gene based on a physiologic rationale. Initial candidates included markers for growth factors, growth factor receptors, protooncogenes, second messengers, contractile proteins, and connective tissue proteins. One hundred and twenty markers were successfully scored, and >28% of the genome was excluded (lod score of −2 or lower) before linkage was identified.

Evidence for linkage was first identified by using the marker *D7S395* (Table 2, ref. 16). In K1773, the lod score was +2.47 at a recombination fraction (Θ) of 0.001. For K1779 the pairwise lod score at this locus was +0.14, again at $\Theta = 0.001$. The combined lod score for both families was +2.61. As *D7S395* had previously been mapped to the long arm of chromosome 7 (7q11), these data suggested that a gene for SVAS was located in that chromosomal region. To improve the statistical support for these findings, we performed linkage studies with two polymorphic markers known to be located near 7q11, *D7S371* and *D7S448* (14, 15). A significant lod score of +4.78 ($\Theta = 0.001$) was identified in K1773 with *D7S448*. Combined lod scores were +1.12 for *D7S371* and +4.65 for *D7S448*, strongly supporting the assignment of a SVAS-encoding gene to chromosome 7q.

We chose *D7S395* as a test marker because it had been mapped to the same chromosomal region as *ELN* (7q11.2, ref. 20), one of many genes that are candidates for SVAS based on a physiologic rationale. To test the hypothesis that

Table 2. Pairwise lod scores for K1773 and K1779

	Recombination fraction					
Kindred	0.001	0.05	0.1	0.2	0.3	0.4
			ELN			
K1773	+5.43	+5.26	+4.85	+3.77	+2.48	+1.03
K1779	+0.50	+0.42	+0.35	+0.21	+0.09	+0.02
Total	+5.93	+5.68	+5.19	+3.98	+2.57	+1.05
			D7S395			
K1773	+2.47	+2.25	+2.03	+1.52	+0.94	+0.35
K1779	+0.14	+0.11	+0.08	+0.04	+0.02	+0.00
Total	+2.61	+2.36	+2.11	+1.56	+0.96	+0.35
			D7S371			
K1773	+0.82	+0.76	+0.69	+0.54	+0.38	+0.20
K1779	+0.30	+0.25	+0.20	+0.11	+0.05	+0.01
Total	+1.12	+1.01	+0.89	+0.65	+0.43	+0.21
			D7S448			
K1773	+4.78	+4.44	+4.04	+3.15	+2.11	+0.95
K1779	−0.13	−0.10	−0.07	−0.03	−0.01	0.00
Total	+4.65	+4.34	+3.97	+3.11	+2.10	+0.95
			D7S8			
K1773	−7.57	−3.47	−2.31	−1.16	−0.56	−0.20

Lod scores (pairwise) between SVAS and five chromosome 7q markers (*ELN*, *D7S395*, *D7S371*, *D7S448*, *D7S8*) in three- and four-generation pedigrees. Lod scores have been calculated, assuming autosomal dominant inheritance with a penetrance of 0.90 for both K1773 and K1779. When penetrance was varied from 0.60 to 0.95, maximum lod scores for K1773 and K1779 combined at $\Theta = 0.001$ ranged from +5.52 to +5.81 for *ELN*, from +3.65 to +4.82 for *D7S448*, from +2.04 to +2.71 for *D7S395*, and from +1.06 to +1.13 for *D7S371*. For purposes of this study the frequency of this rare-disease gene was estimated at 0.001.

ELN is a candidate for the SVAS gene, we performed linkage analysis with a PCR-based polymorphic marker at the *ELN* locus (17). A lod score of +5.43 was obtained for K1773 and +0.50 was obtained for K1779, both at $\Theta = 0.001$. These data confirm the localization of a SVAS gene to the long arm of chromosome 7 and support the candidacy of ELN in the pathogenesis of SVAS.

Multipoint Linkage Data. Although all four markers used in this study have been localized to the same region of chromosome 7 (15–17), marker order is unknown. We attempted to order these loci using the Centre d'Étude du Polymorphisme Humain (CEPH) data base, but marker order could not be determined with certainty, as CEPH mapping data were either incomplete (*D7S371*, *D7S395*, and *D7S448*) or not done (*ELN*). Next we attempted to determine marker order by using data from SVAS families, as these families were typed for ELN. Again, marker order could not be determined with certainty because the families were too small to yield significant marker–marker lod scores >+3. Nevertheless, the best estimate of recombination distance between markers was consistent with linkage (data not shown). The highest lod score was +2.7 at $\Theta = 0.06$ between *ELN* and *D7S448*. We completed multipoint analysis with these two markers and obtained a maximum lod score of +8.4 at the *ELN* locus. This substantial increase in lod score supports the assignment of a SVAS gene to the long arm of chromosome 7.

DISCUSSION

We conclude that a gene for SVAS is located on the long arm of chromosome 7, near *ELN*. We observed no recombination between *ELN* and the disease phenotype, suggesting that *ELN* may be the SVAS gene. Proof of this hypothesis must await further studies, but some existing pathologic data support the candidacy of *ELN*. O'Conner *et al.* (21) examined tissue from six individuals with SVAS; two of these cases were familial, one was a sporadic case, and three had Williams syndrome. These investigators did not discover significant pathologic differences between individuals with different SVAS inheritance patterns. They noted that the medial layer of the aorta in all patients showed a haphazard arrangement of thick elastic fibers, excessive collagen, hypertrophied smooth muscle, and scant ground substance. This tissue contrasts with normal medial tissue, which is highly organized and arranged in parallel layers of connective tissue and smooth muscle. These workers also observed that smooth muscle cells formed clumps or bundles and were the major component of the medial layer. In normal aorta, the most abundant tissue type is elastic tissue. In a study of a single individual with SVAS, Perou (22) also showed that the diseased media contained excessive smooth muscle and reduced elastic tissue. The resulting pattern was that of irregular fascicles of smooth muscle surrounded by fibrous and collagenous tissue with broken, disorganized elastic fibers.

A physiologic rationale for the involvement of *ELN* in SVAS also exists. In addition to its role as a major conduit for blood, the function of the aorta is to absorb energy generated by the heart during systole and release this energy in the form of a sustained blood pressure during diastole. Elastic fibers are responsible for elasticity and resilience of large blood vessels (23). As noted above, the medial layer of the aorta is composed mainly of elastic fibers. Mutations in *ELN*, therefore, might be expected to affect the structure and function of the vasculature, particularly the aorta.

These observations do not prove that *ELN* is the SVAS gene. Pober and colleagues (B. Pober, T. Geva, R. Jonas, R. Lacro, and T. Collins, unpublished data) recently reported a study of seven individuals with SVAS and Williams syndrome, noting that the medial layer of affected aortas contained an increased number of smooth muscle cells, an increased number of normal-appearing ELN fibers, and normal-to-decreased collagen. These investigators also observed an elevated level of platelet-derived growth factor in the tissue and concluded that increased quantities of platelet-derived growth factor stimulate smooth muscle proliferation and cause the cardiovascular abnormalities of SVAS in Williams syndrome (B. Pober, T. Geva, R. Jonas, R. Lacro, and T. Collins, unpublished data). In our study, neither gene for platelet-derived growth factor was linked to the SVAS phenotype (data not shown). Nevertheless, we must consider the possibility that additional candidate genes for this disorder may be identified. The gene encoding an additional connective tissue protein, laminin B1 (*LAMB1*) was mapped several centimorgans telomeric of *D7S395* (24), but we excluded this gene in linkage studies with neighboring markers

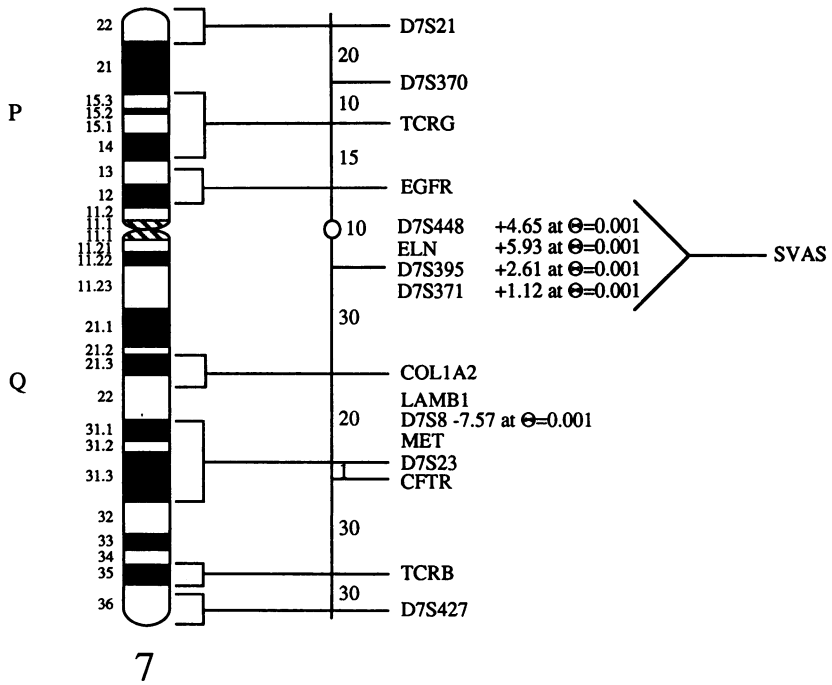

FIG. 2. Approximate map location of the SVAS disease gene. Genes and polymorphic loci mapped to chromosome 7 are shown at right. The approximate genetic distance between these loci in centimorgans is shown on the diagram at center (24). The approximate subsegmental location of these loci is shown on the idiogram at left. In K1773 and K1779, SVAS is linked to *D7S395*, *D7S371*, *D7S448*, and *ELN*, which are mapped approximately to 7q11 between the centromere and *COL1A2* (collagen). SVAS is not linked to *D7S8*, which is located adjacent to *LAMB1* (laminin B1). Pairwise lod scores between a DNA marker and the disease phenotype are indicated.

(Fig. 2). Now that linkage for SVAS has been established, many candidate genes can be excluded based on chromosomal location. Focused mutational analyses of the *ELN* locus will determine whether the SVAS gene is *ELN*.

Because autosomal dominant SVAS and Williams syndrome have dramatic differences in phenotype and because most cases of Williams syndrome are sporadic, some investigators have considered these disorders pathogenetically distinct. With one exception, the affected members of these two families had only SVAS. One member of K1779 had SVAS, learning disability, hoarse voice, and dysmorphic facial features, all characteristics of Williams syndrome. Although individuals with Williams syndrome have rarely been described in SVAS kindreds (2, 25–27), this observation links the Williams syndrome phenotype with the SVAS genotype (Fig. 1*B*). These observations led us to speculate that Williams syndrome and SVAS may be allelic disorders and that the phenotypic differences may be caused by additional genetic factors, environment, or chance. Once the gene for isolated SVAS has been defined, we can test this hypothesis by using mutational analyses.

It is not yet clear whether autosomal dominant SVAS is genetically homogeneous or heterogeneous. No recombination was observed between SVAS and markers on chromosome 7 in either family, suggesting that SVAS is homogeneous in these families. By using the markers described here, it is now possible to test additional families and resolve this issue.

Presymptomatic diagnosis of SVAS can be made in many patients by noninvasive color Doppler-flow echocardiography. Unfortunately, these studies are not completely sensitive, especially in detecting peripheral pulmonary stenoses, which may be difficult to assess in larger patients (4, 28). Invasive cardiac catheterization and angiography are more sensitive, especially in identification of peripheral pulmonary artery stenoses but carry a significant risk of serious complications. In addition, the signs of SVAS may change with time; aortic involvement may worsen while pulmonary findings may improve with age (29–31). The linkage data described here make diagnosis of SVAS in these two families definitive at, or even before, birth. Identification of the gene encoding SVAS will make genetic testing possible for additional families, including sporadic cases. Early, definitive diagnosis will benefit both affected and unaffected family members.

The phenotypic strategy used in this study was similar to one we successfully used in linkage studies of another complex phenotype, the long QT syndrome (32, 33). In both cases, we quantified the results of clinical tests, assigning greater weight to tests with greater diagnostic specificity. Conservative diagnostic criteria were then established, and individuals were classified as affected, unaffected, or uncertain, according to their position in the phenotypic continuum. This strategy may be useful for future studies involving inherited disorders with complex phenotypes.

Vascular disease is one of the more common causes of morbidity and mortality in industrialized societies. Over the last decade, we have learned a great deal about the environmental and metabolic causes of hypertension, hyperlipidemia, and diabetes, important vascular risk factors, but we know relatively little about additional genetic factors that play a role in vascular disease. SVAS and Williams syndrome may offer important genetic clues about the pathogenesis of more common vascular disorders.

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