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Alpha 1 antitrypsin deficiency alleles are associated with joint dislocation and scoliosis in Williams syndrome

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Abstract

Elastin haploinsufficiency is responsible for a significant portion of the Williams syndrome (WS) phenotype including hoarse voice, supravalvar aortic stenosis (SVAS), hernias, diverticuli of bowel and bladder, soft skin, and joint abnormalities. All of the connective tissue signs and symptoms are variable in the WS population, but few factors other than age and gender are known to influence the phenotype. We examined a cohort of 205 individuals with WS for mutations in *SERPINA1*, the gene that encodes alpha-1-antitrypsin (AAT), the inhibitor of elastase. Individuals with classic WS deletions and *SERPINA1* genotypes PiMS or PiMZ were more likely than those with a *SERPINA1* PiMM genotype to have joint dislocation or scoliosis. However, carrier status for AAT deficiency was not correlated with presence of inguinal hernia or with presence or severity of SVAS. These findings suggest that genes important in elastin metabolism are candidates for variability in the connective tissue abnormalities in WS.

Keywords

Williams syndrome; elastin; alpha-1-antitrypsin; *SERPINA1*; scoliosis; joint dislocation; supravalvar aortic stenosis; fibrillin-1; elastic fibers

INTRODUCTION

Williams syndrome (WS) is a multisystem disorder caused by deletion of 26 contiguous genes on chromosome 7q11.23. Haploinsufficiency for the elastin gene in this region accounts for many of the signs and symptoms of the disorder, including the most significant cause of morbidity and mortality, supravalvar aortic stenosis (SVAS). Other phenotypic features of WS related to decreased elastin include hoarse voice, hernias, bowel and bladder diverticuli, and joint abnormalities. All of these phenotypic features have variable severity among individuals with WS. Some of the variability is accounted for by age; e.g., bowel and bladder diverticuli are most commonly observed in adults [Morris et. al., 1990]. Some symptoms vary by gender; e.g., severe SVAS is more common in males than in females [Sadler et al., 2001]. However, these factors do not explain all of the phenotypic variation. To examine the potential modifying relation of other genes to the connective tissue and cardiovascular phenotypes in WS, we elected to examine a common genotype known to be

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associated with variation in elastin metabolism. Alpha-1 antitrypsin (AAT) inhibits elastase; deficiency of this protein is associated with emphysema and liver disease in homozygotes, but heterozygotes also have lower than normal levels of AAT that can adversely impact health. Mutant alleles of the causative gene, *SERPINA1*, are common in the general population, so we reasoned that our cohort of participants with WS would include heterozygotes. The purpose of the study was to determine if mutations in *SERPINA1* influenced the connective tissue phenotypes in WS.

Elastin deficiency is the proximal cause for the connective tissue abnormalities in WS, but other factors likely influence phenotypic variability. Elastin is a major component of elastic fibers that confer elasticity and recoil to several tissues. The organization of elastic fibers depends on the tissue. Elastic fiber networks are found in hollow organs such as the lung, bowel, and bladder, whereas linear arrangements of elastic fibers in association with collagen are typical of ligaments and skin. Concentric rings of elastin are present in arteries and intervertebral discs. In arteries, the elastic fiber rings are interspersed with vascular smooth muscle cells; each ring of elastic fibers and muscle cells forms a lamellar unit. Most elastin is produced in the third trimester of pregnancy and in the first year of life. Turnover is low, but biomechanical stress and resultant inflammation can expose elastic fibers to degradation. Elastase is the primary enzyme that causes elastin degradation.

Alpha 1 antitrypsin (AAT) is a glycoprotein that inhibits elastase, and its deficiency increases the risk for development of chronic obstructive pulmonary disease (COPD) and chronic liver disease [Zorzetto et al., 2008] The genotype for the *SERPINA1* gene determines AAT levels in the serum. The normal gene is labeled PiM and the common deficiency alleles are PiS, which expresses ~50% of normal AAT, and PiZ, which expresses ~20% [de Serres et al., 2003]. The lowest levels of AAT are found with PiZZ and PiSZ genotypes, but diminished AAT levels have been demonstrated in populations with PiMS and PiMZ genotypes [Zorzetto et al., 2008]. The genotype frequencies vary by population; in the USA, the PiMS frequency is 5.88% and PiMZ is 2.78% [de Serres et al., 2003]. Low levels of AAT result in decreased elastase inhibition and therefore increased elastin degradation with inflammation.

There are many connective tissue abnormalities in WS, affecting multiple organ systems. Some signs are nearly universal in WS, such as the hoarse voice that is present in 98% and is due to decreased elastic fibers in the lamina propria of the vocal folds [Hammond et al., 1998, Vaux et al., 2003; Watts et al., 2008]. Other traits present in over 90% include periorbital fullness and full cheeks in infants, lax joints in infancy, and soft skin [Morris et al., 1988]. While joints are loose in infancy, joint contractures typically begin to appear in childhood, worsen with age, and most predominantly affect the lower extremities [Kaplan et al., 1989; Morris et al, 1988; Morris et al., 1990]. The combination of central hypotonia and lax joints in young children contributes to delayed walking. The young child will often develop abnormal compensatory postures, including mild lordosis and kyphosis. The above-mentioned clinical features are present in most individuals with WS, though some vary with age. Elastin deficiency alone is sufficient explanation for occurrence of these traits. Unlike lordosis and kyphosis, however, scoliosis is a fairly low frequency finding in WS, and therefore was one of the traits targeted for investigation in the current study. In a previous study of a cohort of 42 individuals aged 1 – 34 years with WS, 12% had scoliosis, 38% had inguinal hernia, and 64% had SVAS [Morris et al., 1988].

Inguinal hernias are typically diagnosed in infancy in WS and occur in ~40% [Morris, in press] as compared with ~5% incidence in the general pediatric population [Brandt, 2008]. Because decreased elastic fibers in the abdominal wall as a consequence of aging are associated with inguinal hernias in the general population, we investigated the possibility

that AAT deficiency might be associated with hernia occurrence in WS. Bowel and bladder diverticuli are common in WS, but usually present in adulthood, although at an earlier age than in the general population [Pober and Morris, 2007]. Because diverticuli usually are not detected until symptoms warrant invasive investigation, the prevalence in the WS population is unknown and therefore could not be evaluated in this study.

The most serious consequence of the *ELN* deletion in WS is elastin arteriopathy. Aortic smooth muscle cells from individuals with WS produce low levels of elastin mRNA and thus deposit less elastin protein in tissues, resulting in sparse elastic fibers, which in turn results in increased proliferation of smooth muscle cells [Urban et al., 2002]. In the ascending aorta of WS, the lamellar units are increased from the normal number of ~ 40 to ~120, resulting in thickening of the media of the vessel [Dridi et al., 2005]. This change in the number of lamellar units is present at birth [Wagenseil and Mecham, 2009], indicating the *ELN* mutation is the etiology of the abnormality of the media. However, there can be sites of discrete narrowing that occur in areas of hemodynamic stress and turbulence. The proliferation of arterial smooth muscle cells that occurs secondary to elastin deficiency leads to a progression of pathology in that hyperplastic intimal lesions develop [Urban et al., 2002], encroaching on the lumen of the artery. Since these areas would be subject to tissue injury, we hypothesized that the severity of the SVAS may be related to elastin degradation. If so, then lowered AAT levels could be associated with a more severe arterial phenotype. Previous studies have shown that approximately 30% of individuals with SVAS require surgical intervention [Kececioglu et al, 1993].

METHODS

Participants and Clinical Assessment

The study population included 205 individuals with WS (107 females, 98 males), all of whom had the typical deletion on chromosome 7q11.23. Informed consent and specimens were obtained following IRB-approved protocols. Medical records were reviewed on all participants, and physical examinations were performed on 174/ 205 participants by C.A.M. Age at the time of examination ranged from 9 months to 45 years. Inguinal hernia and joint dislocation were scored as present or absent. Scoliosis was assessed by physical inspection including the Adams forward bending test and review of radiography reports. Curves greater than 10 degrees were considered positive for scoliosis. Individuals were coded as negative for scoliosis if they had no physical/radiographic evidence of scoliosis by age 8 years. Children <8 years old were excluded from analysis because they could still develop spinal curvature. None of the participants had vertebral malformations. For individuals who had not had surgery for SVAS, cardiovascular disease severity was scored based on review of echocardiography reports using the recorded Doppler measurements (Table I). Individuals who required surgical correction for SVAS were classified as severely affected.

Genotype Analysis

Cytogenetic preparations were made from either fresh whole blood or previously established lymphoblastoid lines, and standard methods for metaphase FISH experiments to establish the size of deletion were followed as previously described [Hobart et al., 2010, this volume]. All participants had typical deletions of 1.55– 1.8Mb of chromosome 7q11.23.

Genomic DNA was isolated from lymphoblastoid cell lines or whole blood using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) and genotyping was performed by pyrosequencing analysis. The *SERPINA1* Pi Z and Pi S loci were amplified by PCR using the forward primers PiZ 5'-TTGACCTCGGGGGGGATAG-3' and PiS 5'-TTGGTGATGATATCGTGGGTGAGT-3' and reverse primers PiZ 5'(bio)-

GGGATCAGCCTTACAACGTGTC-3' and PiS 5'(bio)-CAATGCCACCGCCATCTT-3'. For each individual, five μl of biotinylated PCR product was analyzed on a Biotage PSQ HS 96 pyrosequencer (Biotage, Uppsala, Sweden) using sequencing primers PiZ 5'-AGCTTCAGTCCCTTTCT-3' and PiS 5'-CGTGGGTGAGTTCATTT-3'. Genotypes were called by the instrument software in SNP analysis mode, and pyrograms were visually checked for errors.

RESULTS

Clinical Assessment

We identified four individuals with WS who had joint dislocations, a finding not previously reported in WS. All 4 individuals in this cohort were heterozygous for AAT mutations. An 18-month- old girl, genotype PiMZ, had dislocation of the left shoulder. A 3.7-year -old girl, genotype PiMS, had bilateral dislocated knees treated surgically. A boy, genotype PiMZ, had a dislocated knee at age 7 years and also was noted to have mild scoliosis. A male, genotype PiMZ, with a history of mild scoliosis had a dislocated right knee treated surgically at age 16 years.

The prevalence of scoliosis in the participants aged 8 years and older was 18%. Most children had a juvenile (between ages 5 and 10 years) presentation of the scoliosis. For some adults in the group, all with mild untreated scoliosis at evaluation, age of onset of scoliosis was not documented. There was no significant difference in the rates of scoliosis between males (13/55) and females (7/56) (Fisher's exact test, $p = 0.145$). Severe scoliosis requiring bracing or surgery was present in 6 participants (Figure 1).

Inguinal hernia (indirect) was diagnosed in infancy in 26% of the participants. A significantly higher proportion of males (38/98) than females (17/107) was affected (Fisher's exact test, $p < 0.0001$).

SVAS was documented in 68%; of those affected, 31.4% required surgical correction. The difference in proportion of males (72/98) and females (68/107) who had any SVAS was not significant (Fisher's exact test, $p = 0.136$). However, a significantly larger proportion of males (29/97) than females (15/107) had severe SVAS (Fisher's exact test, $p = 0.007$).

Genotyping

All participants had the typical WS deletion of 7q11.23 documented by FISH. Observed gene frequencies for each of the possible *SERPINA1* genotypes are reported in Table II. The observed percentage for the MZ genotype was 4.88%. We calculated the 95% confidence interval for this percentage using the modified Wald method [Agresti and Couli, 1998]. The 95% confidence interval (2.56% – 8.85%) included the estimate of 2.78% for the USA previously reported by de Serres et al. [2003] for this genotype. For the MS genotype, the observed percentage was 4.40%. The estimate of 5.88% reported by de Serres et al. [2003] for this genotype is included in the 95% confidence interval for the observed percentage (2.21% – 8.25%). Thus, the observed percentages of the MZ and MS genotypes for the present cohort are consistent with previous estimates for the USA. Genotypes were in Hardy-Weinberg equilibrium, and there were no PiSZ compound heterozygotes observed.

Genotype-Phenotype Correlation

We examined the association between *SERPINA1* genotype and a diagnosis of joint dislocation, inguinal hernia, any SVAS, or severe SVAS for 205 individuals with WS using Fisher's exact tests (Table III). Due to the low prevalence of MZ and MS genotypes, they were combined into a single category of AAT deficiency carriers for analyses. Because the

onset of scoliosis is variable but typically appears in WS prior to age 8 years, only individuals 8 years or older were included in the analysis for scoliosis ($n = 111$). We found that AAT deficiency carriers with WS were significantly more likely than non-carriers to have a diagnosis of joint dislocation ($p < 0.0001$) or scoliosis ($p < 0.0001$).

AAT deficiency carriers with WS were not more likely than non-carriers to have inguinal hernia ($p = 0.171$). However, in contrast to the sex difference found for the full sample, there was no difference in the rate of inguinal hernia for male (4/10) or female (4/9) AAT deficiency carriers (Fisher's exact test, $p = 1.00$).

AAT deficiency carriers also were not more likely than non-carriers to have either any SVAS ($p = 1.00$), or severe SVAS ($p = 0.231$). However, in contrast to the significant sex difference in proportion of individuals with severe SVAS that was found for the full sample, the difference in proportion of male (4/9) and female (2/9) AAT deficiency carriers who had severe SVAS was not significant (Fisher's exact test, $p = 0.620$).

DISCUSSION

As is true with all syndromes, each aspect of the WS phenotype is variable. The connective tissue phenotype is known to be due to decreased elastin production, resulting in diminished elastic fibers in all tissues studied. For traits that are nearly universal in WS, paucity of elastic fibers is sufficient to explain the phenotype. For those features that have a more varied presentation or wide range of severity, other factors are likely contributing to the phenotype.

Elastin Arteriopathy

No effect of *SERPINA1* mutation status was found for the severity of SVAS. While the media of the arterial wall of most medium and large arteries in WS is thick compared to normal, the resultant lumenal narrowing reaches clinical significance only if there is decreased perfusion of an organ, such as may occur in a localized narrowing of the renal artery; or if narrowing above the aortic or pulmonary valve results in outflow tract obstruction that leads to cardiac hypertrophy. Narrowing of the arteries in the pulmonary circulation most typically improves over time, but SVAS may worsen, most commonly in the first 5 years of life [Collins et al., 2010]. SVAS severity is quite variable, with about 30% of individuals who have SVAS requiring surgical treatment, as demonstrated in this study as well as in previously reported series [Collins et al., 2010; Del Pasqua et al., 2009; Kececioglu et al, 1993]. We found an association between severe SVAS and male gender, in agreement with the cross-sectional study of Sadler et al. [2001]. However, gender was not found to be associated with SVAS severity in the recent study of individuals with WS evaluated by a cardiologist before age one year [Collins et al., 2010]. The variability of the SVAS phenotype cannot be explained by gender alone.

While elastase is the primary enzyme responsible for elastin degradation, metalloproteinases (MMPs) are also able to degrade elastin. MMPs increase in tissues in response to inflammation or injury. To investigate the possibility that MMPs are involved in the WS arteriopathy, Dridi et al. [2005] immunolabeled MMPs in aortic tissue samples from WS, isolated SVAS, and controls and found increased staining for MMP2, MMP7, and MMP9 in WS and isolated SVAS compared to controls. However, the tissue samples from affected individuals were removed during aortic surgery, so the individuals had a severe cardiovascular phenotype. Therefore, the increased presence of the MMPs could be primary or secondary to the aortic wall disease. Further investigation of connective tissue physiology in WS and in animal models may provide additional clues into the basis of phenotypic variability.

Inguinal Hernia

Inguinal hernia is more common in WS (38% in a previous series [Morris et al., 1988] and 26% in the current study) than in the general population (~5% [Brandt, 2008]). We found that males with WS were more commonly affected; in the general population the male to female ratio is ~10:1[Brandt, 2008]. Heterozygous mutation in *SERPINA1* was not associated with indirect inguinal hernia in individuals with WS in the current study. Inguinal hernia in adults from the general population is associated with decreased elastin synthesis and increased enzymatic degradation of elastin in the transversalis fascia of individuals with direct inguinal hernia, but not indirect inguinal hernia [Pasqual et. al, 2009]. This finding suggests that AAT mutations could play a role in direct inguinal hernia; however, none of the individuals in our study had this form of hernia.

Scoliosis

Scoliosis, a lateral curvature of the spine, is classified as congenital (most often associated with malformations of the spine such as hemivertebrae), neuromuscular (associated with myopathies, cerebral palsy, etc.), syndrome-related (e.g., Marfan syndrome (MFS), neurofibromatosis type 1), or idiopathic, which is the most common type. Idiopathic scoliosis is further subdivided by age of onset: infantile, 0–4 years, accounting for 0.5%; juvenile, 4–10 years, 10.5%; and adolescent, >10 years, 89% [Janicki and Alman, 2007]. Most individuals with idiopathic scoliosis have mild curvature that does not require treatment. Although 2.5% of the general population has adolescent idiopathic scoliosis (AIS), only .25% of the population requires surgical intervention. The female to male ratio in the surgically treated group is 7 to 1 [Asher and Burton, 2006]. Progression of the curve is most likely to occur during periods of rapid growth.

It has long been observed that scoliosis occurs in families [Miller et al.,1996], and it has been hypothesized that genes involved in connective tissue metabolism may contribute to disease if mutations compromise the biomechanical integrity of the connective tissue. Scoliosis occurs commonly with some syndromes of connective tissue, including MFS, which has an incidence of scoliosis of approximately 60% and is caused by mutations in *fibrillin 1* (*FBN1*). Most individuals with scoliosis who have MFS have mild curves, but there are some who have rapid progression. The cause of the variability of the scoliosis phenotype is unknown. In addition, a three generation family with kyphoscolisis who did not meet the criteria for MFS was found to have a *FBN1* mutation [Ades et al., 2002]. *FBN1* encodes fibrillin 1, a protein that is one of the two major proteins comprising the elastic fiber. The other protein is elastin, which is deficient in WS. Because scoliosis is more common in MFS than in WS, it appears that *FBN1* mutation confers a greater risk for spinal curvature, perhaps due to a dominant negative effect. An alternative explanation is that the unique mechanical properties of elastin and fibrillin 1 account for the differing prevalence of scoliosis in the two syndromes. In studies of the vascular mechanics in mice haploinsufficient for both *ELN* and *FBN1*, Carta et al. [2009] have demonstrated that elastin contributes elastic recoil and fibrillin 1 contributes tensile strength to the aortic wall. If the same principles apply to the intervertebral disc, then fibrillin 1 may have a greater role in accommodating the biomechanical stress occurring during periods of rapid growth.

The intervertebral disc is well organized: Yu et al. [2005] demonstrated that the human anulus fibrosus consists of collagen fiber bundles organized in concentric lamellae alternating with dense elastic fibers around the nucleus pulposis. Elastic fibers are also present within the lamellae, parallel with the collagen bundles. The lamellae of the inner annulus have a denser concentration of elastic fibers that are further organized, oriented at 60 or 120 degree angles to those fibers in the adjacent lamella. This well ordered

architecture is severely disrupted in scolitic intervertebral discs, and elastic fibers are sparse [Yu et al, 2005].

During periods of rapid growth, the spine and its joints—the intervertebral discs—are subject to mechanical force. Tissue injury is associated with increased levels of elastase, which degrades elastin in the extracellular matrix. The findings of the current study indicate that individuals with WS who were heterozygotes for AAT deficiency, and accordingly would have lower levels of AAT and therefore impaired inhibition of elastase, were more likely to develop scoliosis. A similar effect for a different genetic polymorphism has been demonstrated in a case control study of scoliosis in the general population. Aulise et al. [2007] found a higher risk of scoliosis in individuals who had either polymorphisms in the matrix metalloproteinase-3 (MMP-3) gene or polymorphism of the promoter region of the interleukin-6 (IL-6) gene. Both genes are involved in inflammation. The 5A/5A genotype of *MMP-3* conferred a 3.34 odds ratio for scoliosis and the G/G polymorphism of *IL-6* resulted in an odds ratio of 10.54 [Aulise et al., 2007]. Because these polymorphisms are fairly common, studying their associations with severity of scoliosis in populations with abnormalities in extracellular matrix structural proteins, such as MFS and WS, may be fruitful in the characterization of phenotypic variability in those conditions.

Joint Dislocation

An unexpected finding was the association of *SERPINA1* mutations with joint dislocation, a phenotype that had not previously been reported for individuals with WS. Elastic fibers are a major component of ligaments. In large joints, such as the knee, bundles of elastic fibers also comprise a three dimensional network in the articular capsule. Marked reduction in the numbers of elastic fibers in that tissue has been documented in MFS [Gigante et al., 1999]. Joint dislocation has been reported in MFS; investigation of mutations in AAT in MFS may provide insight into the pathogenesis of joint dislocation in that condition.

Summary

In summary, the results from this study suggest that AAT mutation carrier status is not a factor in the severity of the elastin arteriopathy in WS. However, it does appear to have an effect on joints/ligaments. AAT inhibits elastase, which is important in the process of remodeling and repair of the connective tissue. Elastase production increases with inflammation, and an individual with an MZ or MS AAT genotype will have increased degradation of the connective tissue matrix, leading to decreased joint stability. These data add mutations in AAT to the polymorphisms that affect the connective tissue phenotype (scoliosis and joint dislocation) in WS. Future studies should evaluate *MMP-3* and *IL-6* polymorphisms in WS, as these have been associated with scoliosis in the general population. Finally, the results of this study suggest that mutations in AAT should be evaluated in genotype-phenotype studies of other syndromes associated with scoliosis, such as MFS.

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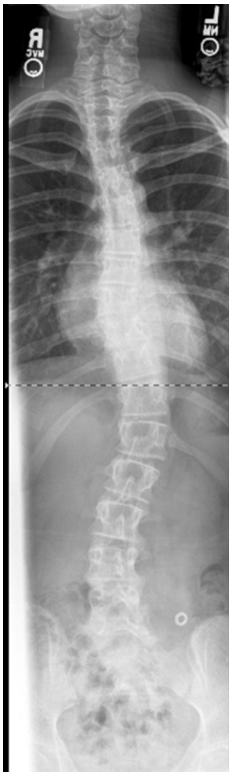

Figure 1.
Spine radiograph of a 12-year-old female with WS and complex scoliosis. The onset of scoliosis was age 6 years, and she currently is treated with a brace. Her AAT genotype is PiMS.

Table I

Classification for SVAS severity based on echocardiography results

Degree of Severity of SVAS	Maximum Velocity (m/s)	Peak Gradient (mm/Hg)
None	<2	<10
Mild	2 – 2.5	11 – 30
Moderate	2.6 – 3.5	31 – 60
Severe	>3.5	>60

Table II

Allele and genotype frequencies of *SERPINA1* PiZ and PiS in Williams syndrome

	Allele Frequency				Genotype Frequency					
	Pi Z		PiS							
	M	Z	M	S	MM	MZ	ZZ	MM	MS	SS
Proportion	400/410	10/410	401/410	9/410	195/205	10/205	0/205	196/205	9/205	0/205
Percentage	97.56%	2.44%	97.80%	2.20%	95.12%	4.88%	0.00%	95.60%	4.40%	0.00%

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Table III

Association of α-1-antitrypsin genotype with connective tissue pathologies in Williams syndrome

	α-1-antitrypsin heterozygotes (MZ or MS)		Normal (MM)			
Diagnosis	**Prevalence**	**Percentage affected**	**Prevalence**	**Percentage affected**	**n**	**p***
Joint dislocation	4/19	21%	0/186	00%	205	<0.0001
Scoliosis	8/11	73%	12/100	12%	111	<0.0001
Inguinal hernia	8/19	42%	47/186	25%	205	0.171
Any SVAS	13/19	68%	127/186	67%	205	1.000
Severe SVAS	6/18	33%	38/186	20%	204	0.231

*From Fisher's exact test

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