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ARTICLE

Identification and characterization of seven novel mutations of elastin gene in a cohort of patients affected by supravalvular aortic stenosis

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Supravalvular aortic stenosis (SVAS) is a congenital narrowing of the ascending aorta, which can occur sporadically as an autosomal dominant condition or as one component of the Williams–Beuren syndrome, a complex developmental genomic disorder associated with cardiovascular, neurobehavioral, craniofacial, and metabolic abnormalities, caused by a microdeletion at 7q11.23. We report the identification of seven novel mutations within the elastin gene in 31 familial and sporadic cases of nonsyndromic SVAS. Five are frameshift mutations within the coding region of the *ELN* gene that result in premature stop codons (PTCs); the other two mutations abolish the donor splice site of introns 3 and 28, respectively, and are predicted to alter splicing efficiency resulting in the generation of a PTC within the same introns of the gene. *In vitro* analysis using minigenes and cycloheximide showed that some selected frameshift mutant alleles are substrates of nonsense-mediated mRNA decay (NMD), confirming that the functional haploinsufficiency of the *ELN* gene is the main pathomechanism underlying SVAS. Interestingly, molecular analysis on patient fibroblasts showed that the c.2044+5G>C mutant allele encodes for an aberrant shorter form of the elastin polypeptide that may hamper the normal assembly of elastin fibers in a dominant-negative manner.
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INTRODUCTION

Supravalvular aortic stenosis (SVAS) has an incidence of 1 in 20 000 live births and was the first disorder to be associated with the *ELN* gene.[1] SVAS may occur as a sporadic disease or it may be inherited in an autosomal dominant manner. It is also classically associated with the Williams–Beuren syndrome (WBS) (OMIM 194050), a complex developmental disorder caused by a microdeletion of ~1.5 Mb of chromosome 7q11.23, which encompasses at least 25 genes, including the *ELN* gene.[2,3] The clinical and structural characteristics of SVAS are identical in both syndromic and nonsyndromic cases. In WBS patients, SVAS is caused by the deletion of one complete copy of the *ELN* gene. In nonsyndromic cases of SVAS, >60 mutations, including substitution, splicing, regulatory, deletion, insertion, and rearrangement mutations, have been identified so far (The Human Gene Mutation Database, www.hgmd.org). Among the point mutations described, there is a prevalence of premature termination mutations that result in null alleles through the nonsense-mediated mRNA decay (NMD) mechanism leading to functional elastin haploinsufficiency.[1,4]

Here, by using DHPLC and directed sequencing of genomic DNA, we identified seven novel *ELN* mutations in a screening of a total of 31 patients mainly affected with familial (13) and sporadic (18) nonsyndromic SVAS, associated in a few cases with other clinical features (Table 1). Functional assay and expression analysis using minigenes and real-time quantitative PCR (QPCR) showed that two selected frameshift mutant alleles are substrates of NMD, confirming that the haploinsufficiency of the *ELN* gene is the main pathomechanism underlying nonsyndromic SVAS. Moreover, of the two *ELN* mutations affecting the splicing process, we tested and showed that the c.2044+5G>C mutant allele results in an aberrant shorter form of the elastin polypeptide, which, by a dominant-negative mechanism, might cause the SVAS phenotype in that family.

MATERIALS AND METHODS

Sample preparation

Patients were enrolled in this study after obtaining appropriate informed consent by the physician in charge and approval of local ethics committees. Patients were recruited from different Italian and Spanish Hospitals (see the

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Table 1 Spectrum of elastin gene mutations

Family patient	*Nucleotide change*	*Location*	*Amino-acid change*	*Clinical phenotype*
SVAS-1	None			PPS
SVAS-2				
I:1	None			Unaffected
II:1	c.304delG	Exon 6	L121X	SVAS, PPS
II:2	c.304delG	Exon 6	L121X	Aortic coarctation
SVAS-4	None			SVAS, aortic coarctation
SVAS-5	None			SVAS, PPS
SVAS-6	None			SVAS
SVAS-7				
I:1	c.1161delC	Exon 20	L463X	SVAS
I:2	None			Unaffected
II:1	c.1161delC	Exon 20	L463X	SVAS
ASD4	None			SVAS, interatrial defect
NW29				
I:1	None			Unaffected
I:2	c.838_839insG	Exon 16	K312X	Nonpenetrant (healthy)
II:1	c.838_839insG	Exon 16	K312X	SVAS
II:2	c.838_839insG	Exon 16	K312X	SVAS
SW67	None			SVAS and ADHD
SW175				
I:2	None			Unaffected
II:1	None			SVAS, MR, and dismorphic features
SW180	None			SVAS and mild facial features
SW181				
I:1	None			Unaffected
I:2	None			Unaffected
II:1	None			SVAS and hypercalcemia
SW184				
I:1	c.40delC	Exon 1	L121X	Unaffected
I:2	None			Unaffected
II:1	c.40delC	Exon 1	L121X	SVAS and mild facial features
SW282				
I:1	None			Unaffected
I:2	None			Unaffected
II:1	None			SVAS
NW37				
I:1	None			SVAS
I:2	None			Unaffected
II:1	None			Unaffected
SW287				
I:1	None			Unaffected
I:2	None			Unaffected
II:1	None			SVAS
SW141				
I:1	c.1939_1940insTG	Exon 27	G675X	SVAS
I:2	None			Unaffected
II:1	c.1939_1940insTG	Exon 27	G675X	SVAS and pyloric stenosis
SVAS-10	None			SVAS
SVAS-11	None			SVAS
SVAS-12	None			SVAS
SVAS-13				
I:1	c.2044+5G>C	Introns 28–29	PTC in introns 28–29	SVAS
I:2	None			Unaffected
II:1	None			Unaffected
II:2	c.2044+5G>C	Introns 28–29	PTC in introns 28–29	SVAS
II:3	c.2044+5G>C	Introns 28–29	PTC in introns 28–29	Nonpenetrant(healthy)
III:1	c.2044+5G>C	Introns 28–29	PTC in introns 28–29	SVAS
SVAS-14				
I:1	None			Unaffected
II:1	None			SVAS
SVAS-15				
I:1	None			Unaffected
I:2	None			Unaffected
II:1	None			SVAS
SVAS-16				
II:1	c.163+2T>C	Introns 3–4	PTC in introns 3–4	SVAS
I:1	c.163+2T>C	Introns 3–4		SVAS

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; MR, mental retardation; PPS, peripheral pulmonary stenosis; SVAS, supravalvar aortic stenosis.
I, II, and III correspond to first, second, and third generation, respectively.

authors' affiliations for recruitment origin of samples). Clinically, all probands in each family presented with SVAS or other vascular abnormalities, such as peripheral pulmonary stenosis, aortic coarctation, and interatrial defects (Table 1). In some cases, there was a family history of SVAS (Table 1). Genomic DNAs were extracted from fresh and frozen blood samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Deletion and mutational analysis

Screening for large *ELN* deletions was carried out by fluorescence *in situ* hybridization (FISH) on metaphase cells following standard technique using LSI *ELN*, D7S486, and D7S522 probes (Vysis, Abbott Laboratories, Abbott Park, IL, USA). At least 30 metaphases for each available sample were analyzed. Putative *ELN* whole-exon deletions were investigated by QPCR (see below).

The entire *ELN* coding sequence was PCR amplified using the set of primers listed in Supplementary Table 1 in 32 amplicons, including complete exons and acceptor and donor splice-site sequences. The nucleotide numbering used in this study follows the coding region of *ELN,* as previously described.[4]

For mutational analysis, we first performed DHPLC analysis using Wave 3500 HT (Transgenomic, Omaha, NE, USA); afterward, when an altered migration profile was detected, purified PCR amplification products were directly sequenced on an ABI3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). All the novel nucleotide variants were further analyzed by segregation analysis in the family patients and by screening 100 healthy unrelated individuals who were used as control samples.

Minigenes generation

From RNA of HeLa cells, we generated a RT-PCR fragment spanning from exon 11 to exon 24 of the elastin gene. We then used DNA of HeLa cells to amplify a genomic fragment of *ELN*, including exon 25, its upstream and downstream intronic sequence, and exon 26. PCR fragments were amplified using the primer listed in Supplementary Table 1. We then ligated the two fragments and cloned them into the pcDNA3.1 vector. This wild-type (wt) construct was used as a template to introduce c.1195delG and c.1161delC mutations by site-directed mutagenesis using the QuickChange II kit (Stratagene, La Jolla, CA, USA). Using similar strategies, we also generated the constructs for c.838_839insG and c.2044+5G>C mutations. Minigenes carrying wt or variant alleles were all verified by sequencing.

Cell transfection and NMD assay

HEK 293 cells were maintained at 37°C in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Invitrogen). Primary skin fibroblasts were obtained from a patient with SVAS and from control subject, and cultured, as previously described.[5]

Nonsense-mediated mRNA decay assays were carried out by transfecting each *ELN* minigene (wt and mutant) into HEK 293 cells (2×10^5 cells per plate) with Fugene HD (Roche Diagnostics, Monza, Italy), according to the manufacturer's instructions. GFP plasmid was used as a reference for transfection efficiency in each experiment. Twenty-four hours after transfection, 300 μg/ml of cycloheximide (CHX) was added. After 4 h, total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the Quantitect Transcription kit (Qiagen), according to the manufacturer's instructions.

The level of mRNA transcribed from different constructs was normalized to the mRNA level of GFP. Then, the ratio between normalized mRNA levels transcribed from the mutant and wt construct after CHX treatment was calculated and compared with this ratio in untreated cells. The experiments were repeated at least thrice.

QPCR

Oligos for QPCR were designed using the Primer3 program[6] with default parameters (primer's sequence available on request). Amplicons and primer pairs were checked both by Blast and Blat against the human genome to ensure specificity. The reactions were run in triplicate in 10 μl of final volume with 10 ng of sample cDNA, 0.3 μM of each primer, and 1× Power SYBR Green PCR Master Mix (Applied Biosystems). Reactions were set up in a 384-well plate format with a Biomeck 2000 (Beckmann Coulter, Milan, Italy) and run in an ABI Prism7900HT (Applied Biosystems) with default amplification conditions. Raw C_t values were obtained using SDS 2.3 (Applied Biosystems). Calculations were carried out by the comparative C_t method.[7] For *ELN* genomic copy number determination, four normalization assays mapping to HSA21 and four normalization DNAs were systematically included in each run as described.[8]

Splice site prediction and statistical methods

Three different splice-site algorithms were used to predict a potential splicing effect:

(http://www.fruitfly.org/seq_tools/splice.html), ASSP (http://www.es.embnet.org/~mwang/assp.html), and NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/).

RESULTS

Mutational analysis

All probands have been diagnosed with vascular anomalies, mainly SVAS (Table 1). FISH and genomic QPCR excluded the presence of *ELN* deletion and/or the partial WBS-deleted region, respectively (data not shown). Mutation analysis of the *ELN* gene in our group of 31 patients enabled us to detect seven novel mutations (Figure 1 and Supplementary Figure 1). Both c.40delC and c.304delG mutations, detected in two unrelated families, result in the same L121X premature termination codon in exon 7. In the SVAS-7 family, we detected a segregating frameshift mutation c.1161delC in exon 20 of the elastin gene. The mutant allele is predicted to encode a 75-amino-acid-long peptide sequence with a premature stop codon (PTC) in the domain encoded by exon 22. Intriguingly, previous studies have shown a clustering of *ELN* mutation in exon 20.[1,5,9] The mutation identified here expands the number of nucleotide variations identified in this putative hot spot mutation region. c.838_839insG was present in two siblings diagnosed with isolated SVAS. Interestingly, the patient's mother carried the same mutation, but she was asymptomatic for SVAS, indicating an incomplete penetrance of the mutation in this family. Finally, the c.1939_1940insTG mutation was detected in a 6-month-old boy and in his father, who also presented with isolated SVAS.

The other two identified base substitutions lie in splice sites of the *ELN* gene.

The c.2044+5G>C transversion was identified five bases downstream from the start of intron 28–29 and it was present in all SVAS-13 family members in whom SVAS had been diagnosed, as well as in one asymptomatic relative. The c.163+2T>C transition was located in the +2 donor splice site of intron 3–4. Both mutations were not seen in 100 healthy unrelated control samples, confirming their absence in the general population.

Expression analysis of *ELN* mutations using minigenes

It has been clearly shown that a number of PTC mutations in *ELN* are substrates of the NMD pathway.[5,10] Therefore, we analyzed the expression level of *ELN* mutant alleles found in SVAS patients and investigated the involvement of the NMD mechanism in the modulation of *ELN* transcript expression. Owing to the impossibility of obtaining primary skin fibroblasts, we devised a strategy on the basis of the use of minigene constructs and transfection assays. We measured by QPCR the *ELN* mRNA levels transcribed by c.1161delC, c.838_839insG, and wt constructs after transfection into HEK 293 cells in the absence and presence of CHX, a widely used inhibitor of NMD. To substantiate the assay, we used a minigene containing the c.1195delG mutation, previously reported as NMD target.[5] The QPCR analysis showed a significant increase in *ELN* mRNA expression for all the analyzed constructs after CHX treatment compared with untreated samples (Figure 2). These results confirm that functional

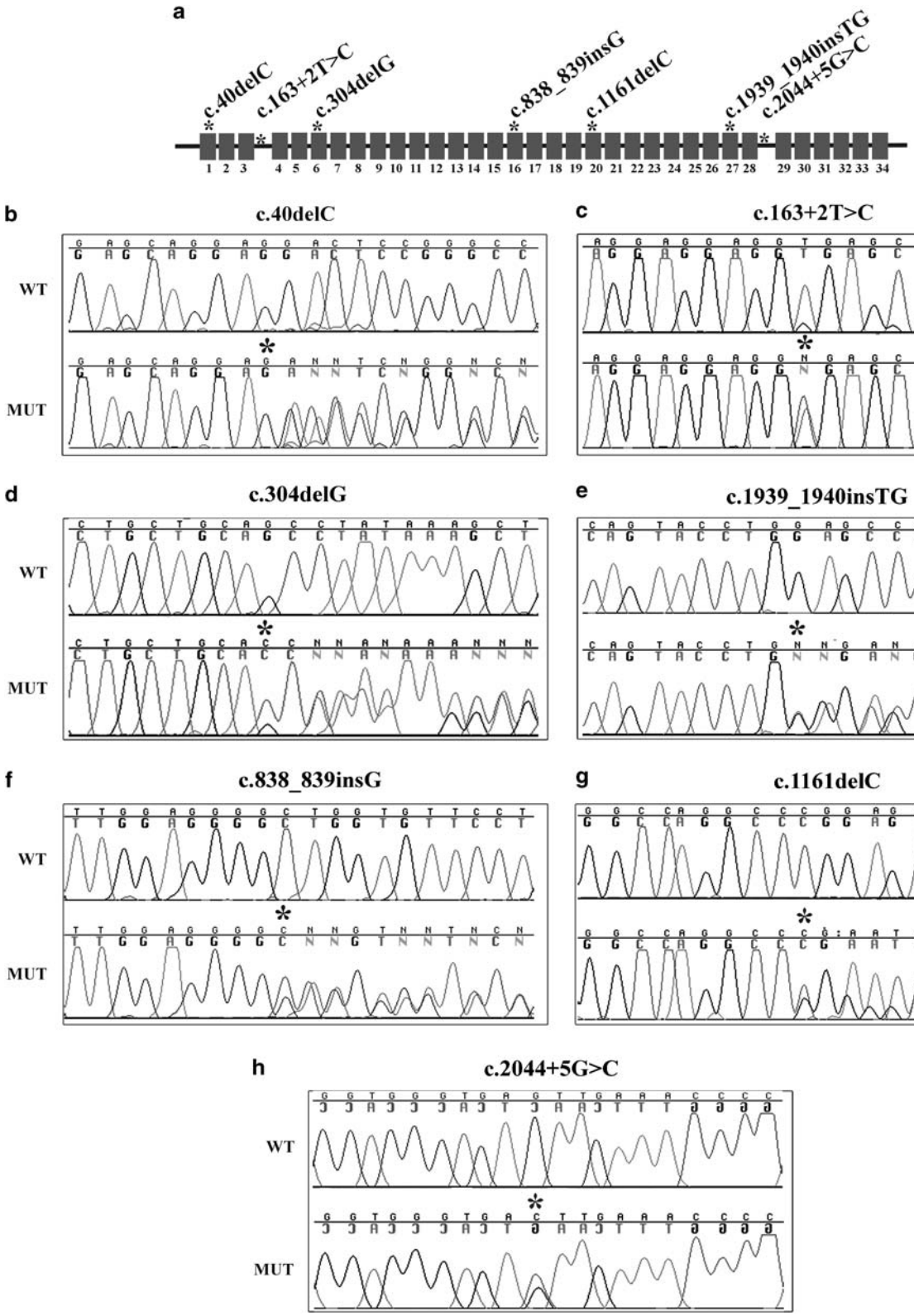

Figure 1 Schematic representation of the *ELN* mutations identified in this study. (**a**) Rectangles represent exons; thin horizontal line represents introns. The schematic position of the identified mutations is indicated with a star. (**b**–**h**) Chromatograms of wild-type and mutant allele for each mutation are showed. Asterisk indicates the position of the mutation.

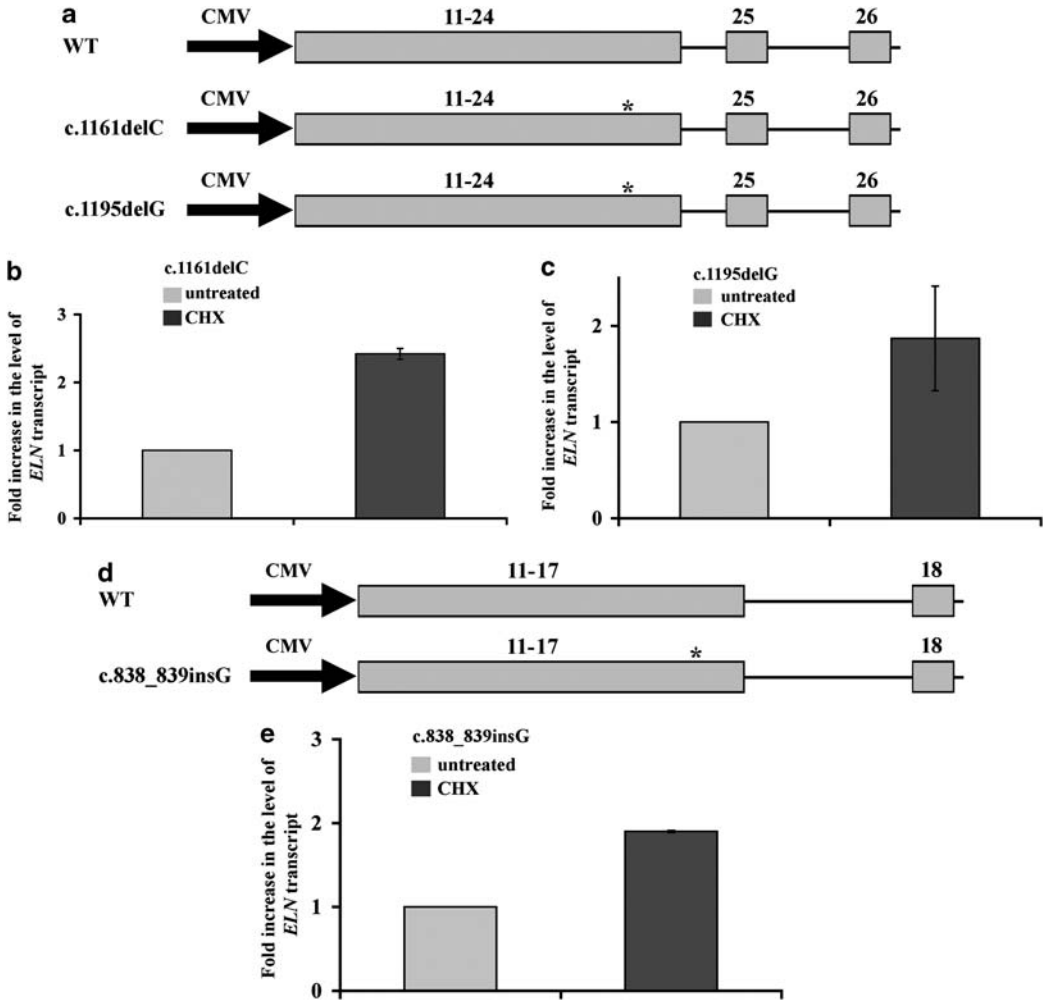

Figure 2 Effect of CHX treatment on the level of *ELN* mRNA. (**a**, **d**) Schematic representation of wild-type, c.1161delC, c.1195delG, and c.838_839insG minigenes, respectively. CMV promoter is marked by a thick horizontal arrow. Boxes indicate coding regions and horizontal thin lines represent introns. The position of mutations is indicated with a star. (**b**, **c**, and **e**) The level of mRNA transcribed from different *ELN* constructs was normalized to the mRNA level of GFP. The ratio between these normalized levels after CHX treatment was calculated and compared with the ratio in untreated cells. The fold increase in the level of indicated *ELN* transcripts is shown as mean ± SEM.

haploinsufficiency of *ELN* through NMD-mediated degradation of aberrant mRNA is one of the primary pathomechanisms leading to SVAS.

Splice-site mutations

The c.2044+5G>C and c.163+2T>C mutations abolish the normal donor splice site of introns 28–29 and 3–4, respectively, as predicted by algorithm analysis.

Hence, we investigated by RT-PCR the consequences of c.2044+5G>C on mRNA splicing using, initially, the minigene-based strategy and then in skin fibroblasts from an SVAS-affected patient and a family relative used as control.

As reported in Figure 3, when we transfected the c.2044+5G>C construct, we detected two distinct mRNA species compared with one transcript of the wt construct (compare lane 4 with lane 3 in Figure 3c). The upper band of about 300 bp corresponds to the aberrant transcript (comprising 30% of normal transcripts), whereas the 200-bp band corresponds to the normally spliced wt mRNA product. Consistently, the same PCR pattern was observed in the skin fibroblasts of the affected patient compared with that of a normal individual (compare lane 4 with lane 2 in Figure 3d). Sequencing analysis of the RT-PCR fragments confirmed that the longer transcript resulted from a splicing failure and inclusion of intron 28–29 in the mRNA, predicting a shorter elastin protein with a PTC within the same intron. Importantly, by semiquantitative RT-PCR, we assessed the *ELN* mRNA expression level after incubating the fibroblast culture of the patient with NMD inhibitor CHX, but we did not find any significant increase in the *ELN* mRNA aberrant transcript, showing that this mutation is conceivably not a substrate of NMD (data not shown).

For the c.163+2T>C mutation, splice sites software predicted a complete exon 3 skipping in the processed transcript, resulting in a frameshift with a stop codon in introns 3–4. Unfortunately, because of the infeasibility of obtaining skin fibroblasts from the patient, the molecular consequence of this mutation was not further investigated.

DISCUSSION

A large spectrum of mutations within the *ELN* locus has been identified as being responsible for nonsyndromic SVAS.[1,4,5,9,11–13] Reduction of *ELN* expression has been reported in the skin fibroblast

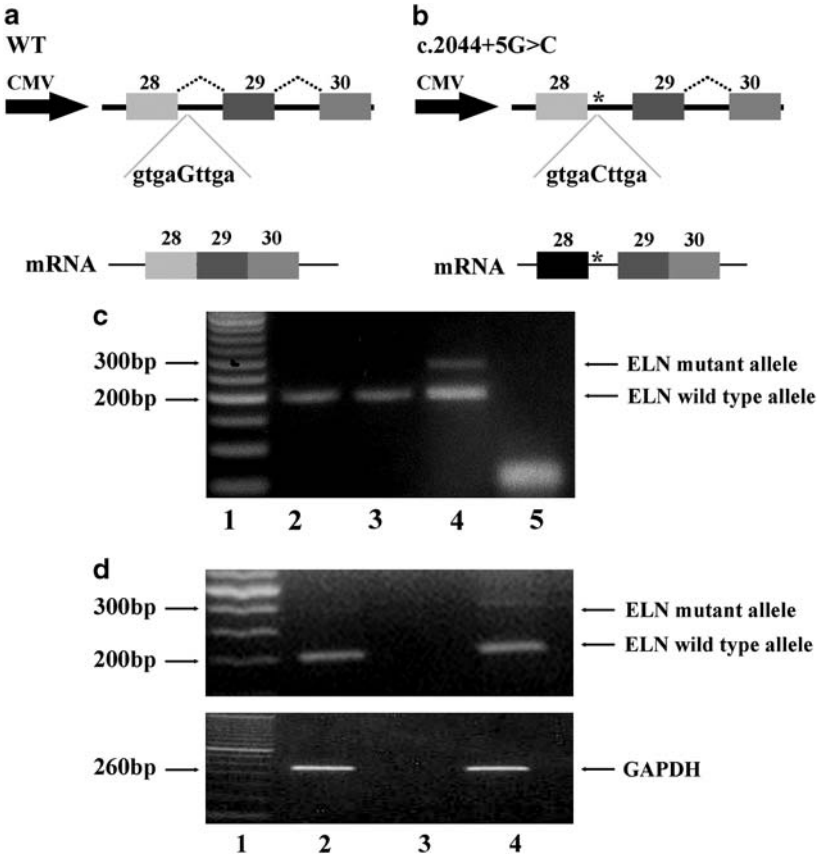

Figure 3 The c.2044+5G>C mutation generates an abnormal *ELN* gene transcript. (**a**, **b**) Constructs containing indicated exons (rectangle box) and introns (black horizontal line) are shown. The position of the c.2044+5G>C mutation is indicated with a star (on the right). The CMV promoter is marked by a thick horizontal arrow. The first nine nucleotides downstream of the exon–intron junction are indicated. Abnormal transcripts generated by splice-site mutation c.2044+5G>C are shown. The PTC position is indicated with a vertical line marked with a star. (**c**) RT-PCR analysis of transcripts produced in HEK293 cells by transfection of normal (lane 3) and c.2044+5G>C (lane 4) minigenes. Lane 1: 50 bp ladder molecular weight marker, lane 2: human cDNA pool, lane 5: negative control. Samples were amplified using oligonucleotides complementary to exons 28 and 29. The uppermost band in lane 4 pertains to a mutant 300-bp transcript containing all intron 28. The bottom band corresponds to the expected transcript of 200 bp. (**d**) RT-PCR analysis of elastin mRNA samples from skin fibroblast cultures of normal (lane 2) and affected (lane 4) individuals.

and aortic smooth muscle cells of SVAS patients, with premature truncation mutations in *ELN* resulting in haploinsufficiency of elastin.[5,10] In an attempt to molecularly characterize our SVAS patient group, we performed a mutation screening of the *ELN* gene. We detected seven novel mutations, of which five result in PTC and two affect the natural splicing of the *ELN* gene.

Consistent with previous studies, our results of mRNA expression analysis of two identified PTC mutations showed that the functional haploinsufficiency of ELN, through nonsense-mediated degradation of mRNA, is one of the primary pathogenic mechanisms leading to SVAS. Even if not formally proven in this study, the other three PTC mutations are also probably targeted by NMD.

Interestingly, in one of the familial cases, the same change was also detected in one of the asymptomatic parents. This was not surprising because it has been reported that disease severity within SVAS families varies from asymptomatic carriers of the mutation to individuals who die in infancy from severe cardiac disease.[1] Variable expressivity and reduced penetrance of elastin arteriopathy are observed in both WBS and nonsyndromic SVAS.[14] This variability is typical of diseases associated with haploinsufciency, in which the genetic background is expected to have a major modifying effect.[4]

To date, only few *ELN* gene alterations reported in SVAS patients are missense mutations.[1,5,12,15] In our group of SVAS patients, we found two point mutations; they induce aberrant and/or additional splicing and are predicted to lead to truncated proteins as well.

We analyzed the effect of the c.2044+5G>C mutation in fibroblast culture and found an aberrant transcript that is predicted to produce a significant amount of abnormal tropoelastin lacking the domains encoded by the last six exons of the gene. The missing part contains several essential hydrophilic cross-linking domains and the well-conserved C-terminus region characterized by two cysteine residues forming a disulfide bond and the positively charged RKRK sequence. It has been shown that the disruption of the disulfide bond dramatically reduces the ability of tropoelastin to assemble and be included in developing fibers, as it does the removal of the C-terminal region.[16] Therefore, we can speculate that the mutant tropoelastin, if secreted from the cell, would likely impair the tropoelastin from assembling in a dominant-negative manner, resulting in a deleterious effect on the normal elastogenesis in those patients. A definitive resolution of this issue would require arterial smooth muscle in order to investigate whether these mutations yield abnormal elastin and/or normal elastogenesis, but this kind of tissue is not available.

Finally, we did not find any *ELN* gene abnormality with our approach in the remaining sporadic and familial patients with clinically diagnosed SVAS included in the study. Familial cases were not investigated for linkage analysis because of the small number of family members available for analysis. DHPLC detection mutation rate and mutations in the regulatory regions of the gene might be the possible causes for the lack of *ELN* mutations in those patients. Alternatively, mutations in another gene could also cause SVAS. In this regard, further studies are required.

In summary, this study illustrates the importance of conducting mutation screening of the *ELN* gene among patients with vascular abnormalities, particularly SVAS and pulmonary stenosis, so that new mutations can be identified and characterized. Our findings also reinforce the idea that haploinsufficiency at elastin is the main cause of these vasculopathies, hence, therapeutic strategies should be directed toward the compensation of this pathogenic mechanism.

KEY POINTS

- Supravalvular aortic stenosis is a congenital narrowing of the ascending aorta, which can occur sporadically as an autosomal dominant condition or as one component of WBS.
- We identified seven novel mutations within the elastin gene in a group of familial and sporadic cases of nonsyndromic SVAS.
- This study reinforces the idea that haploinsufficiency at elastin is the main cause of these vasculopathies and illustrates the importance of conducting mutation screening of the *ELN* gene among patients with vascular abnormalities, particularly SVAS and pulmonary stenosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)