Novel Types of Mutation Responsible for the Dermatosparactic Type of Ehlers-Danlos Syndrome (Type VIIC) and Common Polymorphisms in the *ADAMTS2* Gene

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Abstract

Ehlers-Danlos syndrome (EDS) type VIIC, or dermatosparactic type, is a recessively inherited connective tissue disorder characterized, among other symptoms, by an extreme skin fragility resulting from mutations inactivating ADAMTS-2, an enzyme excising the aminopropeptide of procollagens type I, II, and III. All previously described mutations create premature stop codons leading to a marked reduction in the level of mRNA. In this study, we analyzed the *ADAMTS2* cDNA sequences from five patients displaying clinical and/or biochemical features consistent with a diagnosis of either typical or potentially mild form of EDS type VIIC. Three different alterations were detected in the two patients with typical EDS type VIIC. The first patient was homozygous for a genomic deletion causing an in-frame skipping of exons 3-5 in the transcript. In the second patient, the allele inherited from the mother lacks exon 3, generating a premature stop codon, whereas the paternal allele has a genomic deletion resulting in an in-frame skipping of exons 14-16 at the mRNA level. Although the exons 3-5 or 14-16 encode protein domains that have not been previously recognized as crucial for ADAMTS-2 activity, the aminoprocollagen processing was strongly impaired *in vitro* and *in vivo*, providing evidence for the requirement of these domains for proper enzyme function. The three other patients with a phenotype with some resemblance to EDS type VIIC only had silent and functionally neutral variations also frequently found in a normal population.

Keywords : ADAMTS2 ; dermatosparaxis ; Ehlers-Danlos Syndrome ; genetic skin disease ; procollagen peptidase

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of inherited connective tissue disorders characterized by altered mechanical properties of skin, joints, blood vessels, and ligaments (Beighton *et al*, 1998; Steinmann *et al*, 2002). EDS type VIIC (MIM 225410) (Nusgens *et al*, 1992; Smith *et al*, 1992; Fujimoto *et al*, 1997) and the related animal disease (dermatosparaxis) (Hanset and Ansay, 1967) are recessively inherited connective tissue disorders. They result from a defect in the processing of type I procollagen to collagen, with accumulation in most tissues of molecules that retain the amino-terminal propeptide (Lenaers *et al*, 1971). These precursor molecules self-assemble into abnormal ribbon-like fibrils that fail to provide normal tensile strength to tissues

(Piérard and Lapière, 1976; Nusgens *et al*, 1992). The defect in the processing of type I procollagen reflects the absence of functional ADAMTS-2, an enzyme removing the aminopropeptide of type I, type II, and type III procollagen (Lapière *et al*, 1971; Colige *et al*, 1995, 1997; Wang *et al*, 2003). ADAMTS-2 is a metalloproteinase of the ADAMTS family containing, among other features, a Zn^{2+}-binding catalytic site required for its procollagen processing activity, and four thrombospondin type I repeats (TSPI) most probably crucial for the recognition of the substrate and the 3D-structure of the protein. Three different mutations have been reported so far in the *ADAMTS2* gene (Colige *et al*, 1999). They generate stop codons resulting in a marked decrease of the level of mRNA.

In this study, the cDNA and the genomic DNA of two patients displaying a typical EDS type VIIC phenotype (P7 and P8) were analyzed. We showed that two of the three mutations in these new patients permit stable mRNA to accumulate and predict that they lead to synthesis of proteins that lack activity or are degraded. In addition, in order to identify potential mutations affecting the enzyme activity less severely, analyses were performed also on cDNA from patients presenting only some of the clinical signs of the disease (cases 1-3).

Results

Mutation detection

The *ADAMTS2* cDNA from the five patients and from control individuals were PCR-amplified using five primer pairs and separated on agarose gel. Normal amplification products were obtained for all samples except for product 1 (undetectable) and 2 (± 450 bp smaller than expected) from P7 and product 4 (± 390 bp smaller than expected) from P8 (Fig 1*A*). Sequencing of the purified products showed that these alterations resulted from the absence, at the cDNA level, of bases 535-975 for P7 and bases 2086-2457 for P8, + 1 being the A of the ATG start codon (Fig 1*B*). Determination of the exon/intron structure of the *ADAMTS2* gene was required for the identification of the molecular defect leading to these deletions. Since the human genome sequence containing the *ADAMTS2* gene was not yet available, three overlapping clones covering the entire gene were isolated from a human genomic DNA BAC library. By FISH, PCR, and sequencing, it was demonstrated that the gene, located on chromosome 5 (5q35), contains 22 exons (Fig 1*B*) separated by 21 introns of various sizes (from 425 bp to >50 kbp). The sequence absent from the cDNA of P7 corresponded exactly to exons 3-5 (Fig 1*B*). PCR amplifications of exon/intron junctions (± 200 bp fragments) in this region of the gene (E2-I2, I2-E3, E5-I5, and I5-E6) was performed (Fig 2*A*). For P7, PCR products were obtained using primer pairs designed to amplify the E2/I2 and I5/E6 borders but not with primers specific for the I2/E3 or E5/I6 borders, indicating that the in-frame skipping of exons 3-4-5 is caused by a deletion starting in the ± 70 kbp intron 2 and ending in the ± 25 kbp intron 5. The exact border of the deletion was not determined. Analysis of the cDNA of his parents was also performed, using a forward primer in exon 2 and a reverse primer in exon 7. PCR product amplified from the WT sequence was observed for the control and the parents but not for P7, confirming his homozygous status for the deletion (Fig 2*B*). The DNA product corresponding to the E3 to E5-deleted allele was obtained from P7 but was also present in both parents, although at a very low level as illustrated by comparison of the amount of the two PCR products amplified after 28 and 31 cycles. In order to confirm the presence of the deleted allele in parents, a deletion-specific PCR was set up (Fig 2*C*). It consisted in the use of a forward primer in exon 2 and a reverse primer designed to be specific for the exon 2-exon 6 fusion sequence, a sequence found only in the deleted cDNA. PCR product was obtained for P7 and his parents but not for the control, confirming the data shown in panel B.

Figure 1. *Mutation analysis. (A) RNA from the patients and from a normal control were reverse transcribed and amplified using five different primer pairs generating overlapping products covering the entire ADAMTS2 cDNA. Localization of the five products is given relative to the exon structure of the cDNA. Only the products 1, 2, and 4 from P7, P8 and the normal control are shown here. For P7, PCR product 1 was absent and the size of PCR product 2 was smaller than expected. For P8, product 4 was of smaller size as compared to other samples. These data suggest the presence of a deletion in the region of exons 3-5 for P7 and of exons 14-16 for P8. (S) Schematic representation of the exon structure (exons 1-22) of the coding sequence of the ADAMTS2 cDNA. The localization of the sequence polymorphisms and mutations in P7 and P8 are reported, with A of the ATG start codon being referred to as +1. Mutations causing the disease in patient 1 (P1), in patients 2-6 (P2 → 6) and in the dermatosparactic calves (D calf) have been reported earlier (Colige et al, 1999). (C) The upper part of the panel represents the functional domains of a normal ADAMTS-2 enzyme. The double arrows indicate the part of the enzyme which is synthesized in the patients and in the dermatosparactic calf, on the basis of the presence of either premature stop codons or deletion at the mRNA level, but without considering the effect of nonsense-*

mediated decay process. For P7 and P8, the dotted line represents the missing protein sequence resulting from the exon skipping. D calf and P1-P7 are homozygous for their respective mutation, whereas P8 is a compound heterozygous explaining why the product from the two alleles is represented.
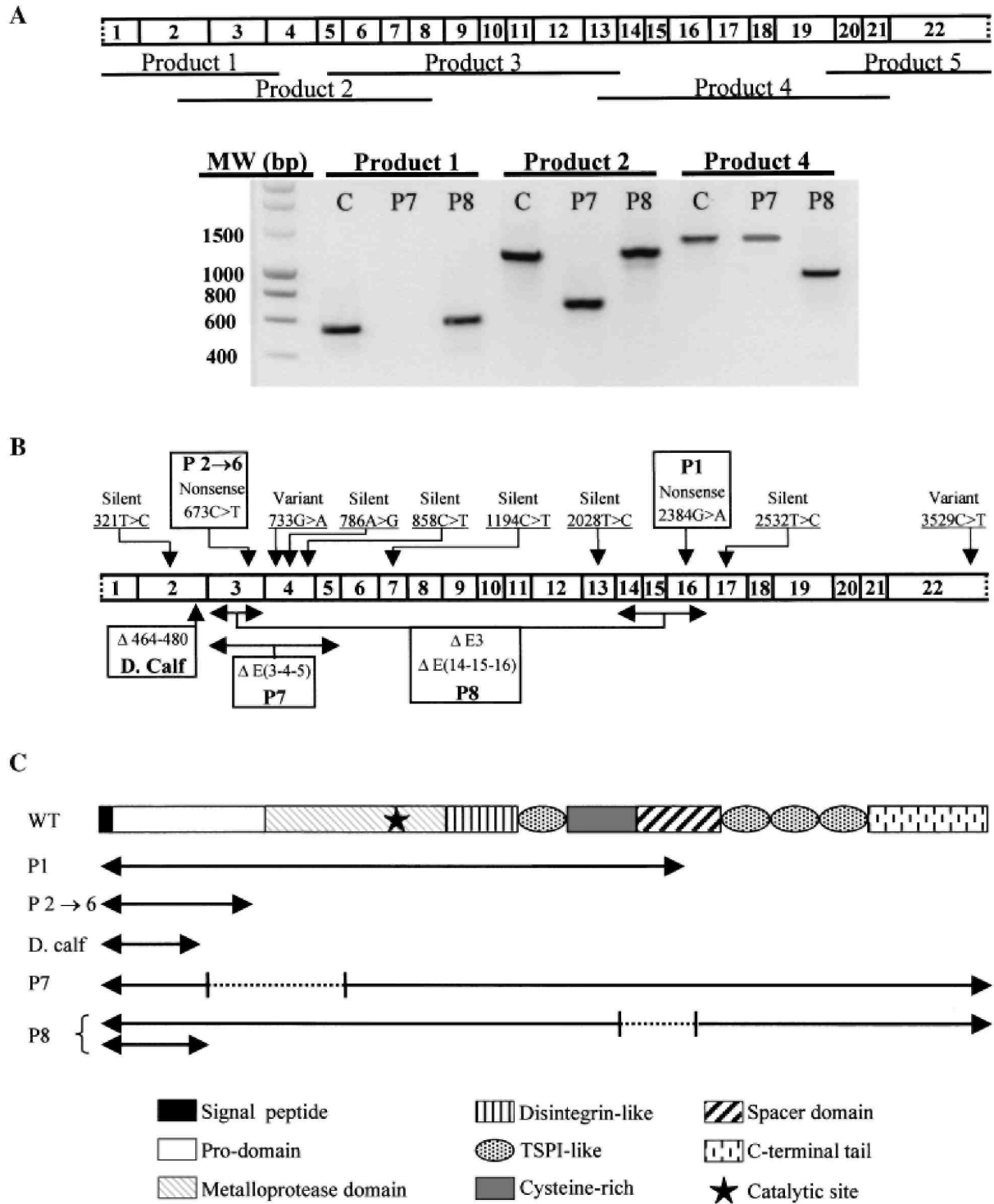

Figure 2. *Genomic DNA analysis and homo/heterozygosity in the family of P7. (A) PCR amplification of genomic DNA from P7 and a control (C) was performed using primers specific for various exon/intron borders in exons 2-6 part of the ADAMTS2 gene. For P7, PCR products were obtained using primer pairs designed to amplify the E2/I2 and I5/E6 borders but not with primers specific for the I2/E3 or E5/I6 borders, indicating that the in-frame skipping of exons 3-4-5 is caused by a deletion starting in intron 2 and ending in intron 5. (B) PCR amplifications of cDNA from a control (C), P7 and his parents (father: FP7; mother: MP7), and P8 were*

performed, using 28 (28#) and 31 (31#) cycles, with a forward primer in exon 2 and a reverse primer in exon 7. Product of normal size (WT) was amplified for the control and the parents, whereas the product lacking exons 3-5 (Δ, E3-E5) was obtained for P7 and his parents. (C) Allele-specific PCR was performed using a forward primer in exon 2 and a reverse primer designed to be specific of the exon 2/exon 6 fusion sequence (3'-CCGAC/AGGTACTCGGAG-TAGCTC-5'). PCR product was obtained only for P7 and his parents, confirming the homo/heterozygosity status suggested by data shown in panel B.
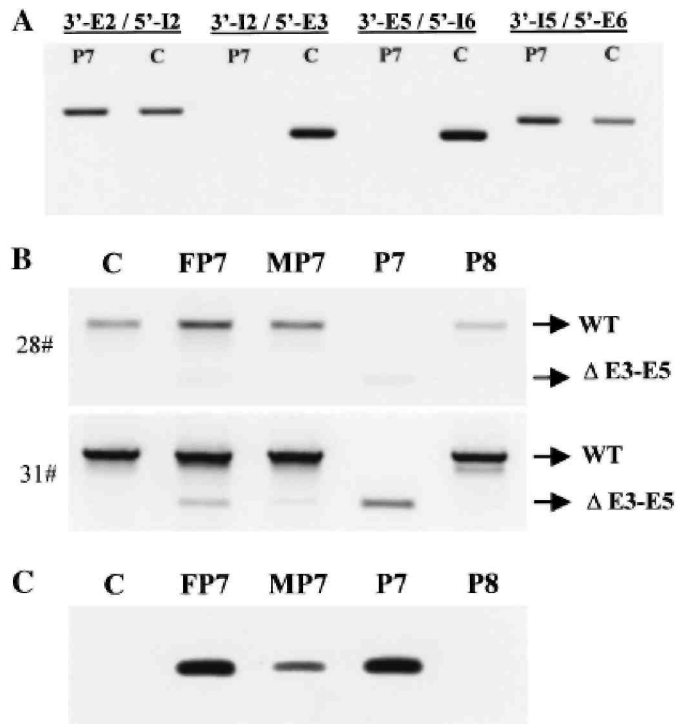
For P8, sequencing of product 4 (Fig 1*A*) indicated that the sequence absent at the cDNA level corresponds to exons 14-16 (Fig 1*B*). In order to elucidate the cause of the skipping of these exons, the cDNA and the genomic DNA of the patient, his parents and a normal control were further analyzed. PCR amplification of genomic DNA using primer pairs located on exon 13 and 17 yielded only one band of the expected size (7.9 kbp) for the mother and the control and two bands for P8 and his father, suggesting the presence of a 5.9 kbp deletion (Fig 3*A*). Sequencing of the 2 kbp product revealed that the entire region from base 1422 of the 3008-bp intron 13 to base 1335 of the 1813-bp intron 16 was skipped and that a TCC triplet insertion occurred at the deletion junction. PCR amplification of cDNA using primers located on exon 13 and exon 18 showed only the WT product for the control and the mother of P8 whereas, for the father, WT and deleted product were present and displayed similar intensities (Fig 3*B*). For P8, the WT product was also amplified but at a very low level requiring 31 cycles of amplification for visualization. These results suggested that P8 inherited the exons 14-16-deleted allele from his father and the other allele from his mother. This maternal allele should contain a premature stop codon leading to an efficient process of nonsense-mediated decay explaining why the message produced from this allele can barely be seen here (Fig 3*B*) and was not initially detected by RT-PCR (Fig 1*A*, product 4, P8). In order to identify the mutation affecting the maternal allele of P8, two primer pairs were designed, one with the forward primer in exon 1 and the reverse primer in exon 16 and the second one with the forward primer in exon 15 and the reverse primer in the 3'-untranslated sequence. Since exons 15 and 16 are absent from the paternal allele (Fig 1*B*), this strategy allowed to specifically amplify the maternal allele. Sequence of this maternal-amplified DNA revealed that the sequence corresponding to exon 3 is absent. This leads to a frame shift, generating a new amino acid sequence from the beginning of exon 4 (GPPWTAWTASAAPWAS) followed by a premature stop codon upstream of the sequence that normally codes for the catalytic site of the enzyme. As a confirmation, RT-PCR amplification of the *ADAMTS2* cDNA using a forward primer in exon 2 and the reverse primer in exon 4 shows two bands for P8 and his mother, the smallest one being the product lacking exon 3 (Fig 3*C*). The strong difference in the level of the two products clearly illustrates an active process of nonsense-mediated decay affecting the message containing the premature stop codon. Presence of the allele lacking exon 3 was also illustrated by using another primer pair (Fig 2*B*, P8).

Analysis of the cDNA from cases 1-3 revealed two differences compared to the published sequence: a G to A transition (733G → A) changing a codon for valine into a codon for isoleucine (Val225Ile) and a C to T transition (3529C → T) changing a codon for proline into a codon for serine (Pro1177Ser) (Fig 1*B*). Case 1 was heterozygous at these two positions (Val/Ile and Pro/Ser) whereas case 2 (Ile/Ile and Ser/Ser), P7, P8, and case 3 (Val/Val and Pro/Pro) were homozygous. A similar study was performed in a normal population (50 unrelated individuals). The calculated allele frequency was 70/30 for G/A at position 733 and 62/ 38 for C/T at position 3529.

Figure 3. *Genomic DNA analysis and homo/heterozygosity in the family of P8. (A) PCR amplification of genomic DNA from P8, his parents (father: FP8; mother: MP8), and a control (C) was performed using a primer pair allowing the amplification of the part of the ADAMTS2 gene extending from exons 13-17. Only the product of expected size (WT: 7.9 kbp) was amplified for the mother and the control whereas two bands (WT: 7.9 kbp and Δ: 2 kbp) were observed for P8 and his father, suggesting the existence of a 5.9 kbp deletion. (B) PCR amplifications of cDNA from a control (C), P8, and his parents (father: FP8; mother: MP8) were performed, using 28 (28#) and 31 (31#) cycles, with primers located on exons 13 and 18. The full size product (WT) only was amplified from cDNA of the control and the mother of P8. For the father, the WT and deleted product (Δ, E14-E16) were both detected. In contrast, the WT product amplified from P8 was almost undetectable (arrow). (C) PCR amplification of cDNA from a control (C), P8, and his parents using primers located on exons 2 and 4. The full size product (WT) was observed for all samples whereas the product lacking exon 3 (Δ, E3) was seen only in samples from P8 and his mother.*
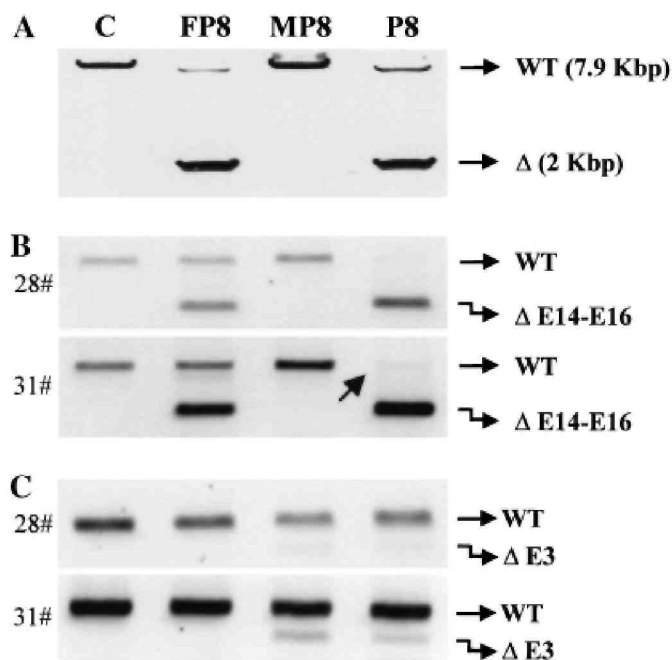
Ultrastructure of dermal collagen fibrils and procollagen processing

In normal skin, collagen fibrils appear cylindrical in cross section and elongated with a typical banded pattern in longitudinal section (Fig 4*A* and *B*). In the skin of P7 and P8, fibrils were hieroglyphic in cross section and frayed and disorganized in longitudinal section (Fig 4*D-F*). This pattern was identical to the pattern observed in other EDS VIIC patients, in the dermatosparactic calf and in ADAMTS-2 deficient mice (Fig 4*C*) and clearly different from the pattern observed in EDS VIIA and B (Byers *et al*, 1997). Collagen synthesis and processing were also evaluated in the culture of fibroblasts grown from skin biopsies (Fig 5). Analysis of the collagen secreted in the culture medium of fibroblasts of P7 and P8 revealed an almost complete absence of fully processed type I chains (α1 and α2) and some accumulation of type I aminoprocollagen (pNα1 and pNα2), evidencing a strong reduction of aminoprocollagen peptidase activity. As another consequence of this altered enzymatic activity, type I carboxyprocollagens (pCα1 and pCα2) are not found since, in absence of aminoprocollagen processing, the only procollagen polypeptides retaining the C-propeptide are α1 and α2

procollagens (pro α1 and pro α2). For cases 1 and 2, similar but milder alterations of the procollagen profile were observed, suggesting a mild reduction of the aminoprocollagen I peptidase activity, whereas the processing determined for case 3 was similar to that observed with control cell strains.

Figure 4. *Structure of collagen fibrils. Collagen fibrils from skin of human (A, D, E, F) and mouse (B, C) were studied by transmission electron microscopy. In normal skin (A, B), collagen fibrils appear cylindrical in cross section and elongated with a typical banded pattern in longitudinal section. In the animal model for the disease (C, AD-AMTS2 null mouse), and in EDS type VIIC patients (D: P7; E, and F: P8), fibrils are hieroglyphic in cross section and disorganized in longitudinal section. The level of observed hieroglyphic pattern is influenced by the angle of the section and can vary slightly between different regions within a single skin biopsy (C-F). Scale bar =0.3 μm.*
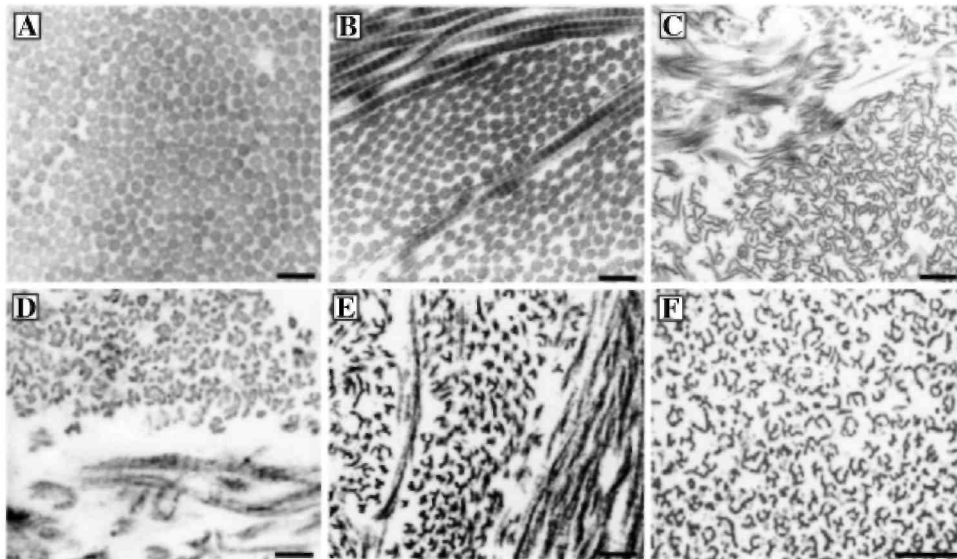

Figure 5. *Electrophoretic pattern of collagen polypeptides. Fluorograms of 14C-proline-labeled collagen polypeptides collected from the culture medium of skin fibroblasts of two normal control individuals (Cont A and B), of P7 and his parents (MP7, FP7), P8 and his parents (MP8 and FP8), and Cases 1-3. The right and left panels correspond to two different labeling experiments. The various type I collagen polypeptides are indicated. The two bands, above and under the proα1 band, are proα1 III and pNα1 III. Although some variability in the processing of type I procollagen by normal human fibroblasts can exist (compare Cont A and B), the illustrated control patterns are representative examples. Note in P7 and P8 the lack of processing of proα1 and proα2 into the C-terminal extended polypeptides (pCα1 and pCα2); the near absence of fully processed α1 and α2 chains and some accumulation of pNα1 and pNα2, all of these features being characteristic of decreased aminoprocollagen peptidase activity. For cases 1 and 2, similar but milder alterations of the procollagen profile were observed, whereas the processing determined for case 3 was similar to that observed in control cell strains.*
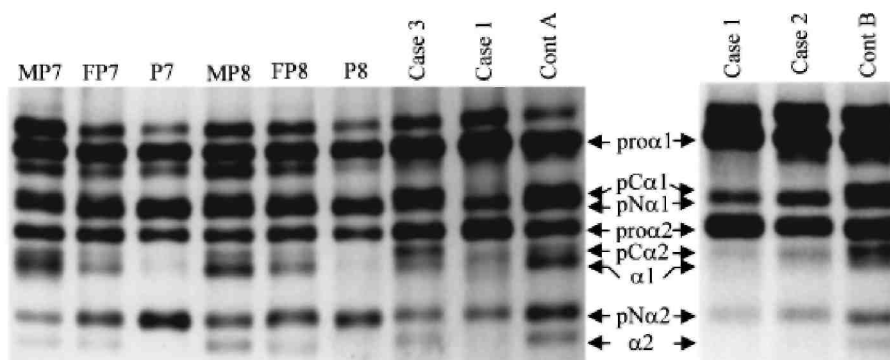
Discussion

Ehlers-Danlos type VIIC is a rare inherited connective tissue disorder caused by mutations affecting the gene for ADAMTS-2, the enzyme responsible for the excision of the aminopropeptide of type I procollagen. To date, three different mutations generating a premature stop codon have been reported: one in the calf, the second in one Belgian patient and the third in five unrelated patients of various origins (Colige *et al*, 1999).

In this study, the presence of a mutation affecting the *ADAMTS2* gene was searched in five additional patients. Two of them, P7 and P8, had highly fragile skin and most typical hallmarks of EDS type VIIC. The three other patients, designated as cases 1-3, displayed only some of the features typical of the disease. For P7 and P8, the EDS type VIIC diagnosis was confirmed by *in vitro* biochemical studies, showing the near absence of fully processed α chains and accumulation of α1 and α2 aminoprocollagen I, and by evaluation of the shape of type I collagen fibril *in vivo*, showing the typical hieroglyphic pattern characteristic of EDS type VIIC. Besides the different clinical features, these observations excluded the diagnosis of the related EDS type VIIA or B, that would have caused an accumulation of either α1 or α2 aminoprocollagen I chain and resulted in a less dramatic alteration of the pattern of collagen fibrils (Byers *et al*, 1997). P7 is homozygous for a genomic deletion inherited from his heterozygous parents. This deletion, starting in intron 2 and ending in intron 5, results in the absence of exons 3-5 from the *ADAMTS2* mRNA and is probably responsible for the very low level of the altered mRNA. If the small amount of mRNA lacking exons 3-5 can be translated, it would result in the production of low levels of an enzyme still containing the catalytic site and the entire downstream sequences but lacking the end of the pro-domain and the beginning of the metalloprotease domain (Fig 1*C*). This region of the enzyme does not contain Cys residues which are often important for the 3D-folding of proteins but does contain a cleavage site by furin which is crucial for the activation processes of many enzymes including ADAMTS (Rodriguez-Manzaneque, 2000). Therefore, the defect in aminoprocollagen-processing activity in this patient may result from the low level of ADAMTS-2 and/ or the absence of activation process of ADAMTS-2 at the furin cleavage site.

P8 is the first example of an EDS type VIIC caused by compound heterozygosity. In the allele from the mother, deletion at the mRNA level of exon 3 resulted in a frame shift, generating a premature stop codon upstream of the catalytic site and a very efficient process of nonsense-mediated decay. Identification of the molecular defect causing the skipping of exon 3 was not successful, because the size of introns 2 and 3 (about 70 kbp each) did not allow the PCR amplification of this entire region of the gene and because the compound heterozygous status prevented identification of a deletion by using exon 3-specific primers. In the allele inherited from his father, a genomic deletion extending from intron 13 to 16 results in an in-frame deletion of exons 14-16 at the mRNA level. Although this region of the gene does not encode domains known to be important for the ADAMTS-2 function, this deletion clearly affected the enzyme activity *in vivo* as demonstrated by the alteration of collagen fibrils. The specific role of this part of ADAMTS-2 is not yet elucidated but we speculate that this region might be involved in the correct folding of the protein, in the adequate spacing between the thrombospondin type I repeats or in the recognition of the substrate. Similar requirement for enzymatic activity of the various C-terminal domains, including the Cys-rich and the spacer regions, have been reported for other ADAMTS enzymes such as ADAMTS-1, -4, -9 and -13 (Kuno *et al*, 1998; Tortorella *et al*, 2000; Somerville *et al*, 2003; Zheng *et al*, 2003). The delayed onset of a typical phenotype for P8 highlights the fact that a correct diagnosis of EDS type VIIC cannot always be made at birth, but sometimes requires follow-up of the patient during the first years of life. We cannot rule out the possibility that this slightly different phenotype reflects the presence of residual activity, possibly developmentally regulated, of ADAMTS-2 lacking the region encoded by exons 14-16. Since another ADAMTS, ADAMTS-13, can process von Willebrand factor (George *et al*, 2002), another intriguing feature for this patient was the presence of a mild form of von Willebrand disease type I. Since no overlap has been described so far between ADAMTS-2 and ADAMTS-13 in terms of tissue localization and synthesis, mechanism of activation and substrate specificity, the occurrence of the two diseases in one patient may be fortuitous. Reappraisal of von Willebrand factor levels in the other EDS type VIIC patients would help to elucidate this point.

For cases 1-3, clinical diagnosis was not suggestive of a typical EDS type VIIC because these patients display only some of the features of the disease. The pattern of collagen polypeptides secreted by the fibroblasts in culture was characterized and the relative abundance of aminoprocollagen and fully processed α chains was determined as a mean to evaluate the aminoprocollagen peptidase activity. For case 3, the collagen pattern was normal and no mutation was detected in the *ADAMTS2* cDNA. For fibroblasts from cases 1 and 2, a significant reduction of the procollagen processing was observed, that could have been caused by mutations only mildly affecting ADAMTS-2 activity. Sequencing of the cDNA revealed two differences as compared to the published sequence. Screening of 50 cDNAs, however, from normal individuals showed that these modifications are

relatively common and should be considered as polymorphisms that do not affect the ADAMTS-2 function. ADAMTS-3 and -14 are two other enzymes displaying aminoprocollagen peptidase activity (Femandes *et al*, 2001; Colige *et al*, 2002). On this basis, mutations in these genes could be the cause of the clinical phenotype of cases 1 and 2. The level of expression of ADAMTS-3 in skin and tendon is very low, strongly suggesting that it is not significantly involved in the aminoprocollagen I processing *in vivo* in these tissues. No further investigation about ADAMTS-3 was performed. Because of its relatively high level of expression, the cDNA of ADAMTS-14 was sequenced but no mutation was identified (unpublished data). Data about cases 1 and 2 suggest that the reduced aminoprocollagen peptidase activity measured *in vitro* may be due to a mutation in another protein that would be required for optimal ADAMTS-2 function. Such a potential co-factor has not been identified yet. Observations reporting that native ADAMTS-2 can be isolated as a complex with other proteins (Hojima *et al*, 1994), however, would support this hypothesis.

In this study, we describe three novel mutations affecting the *ADAMTS2* gene and causing EDS type VIIC in two patients. Skipping of exons 3-5 from the mRNA of patient P7 resulted in a typical EDS type VIIC phenotype that can be diagnosed at birth, as in previously reported EDS type VIIC patients (Colige *et al*, 1999). Mono-allelic in-frame skipping of exons 14-16, in association with another allele producing a totally inactive protein, as observed for P8, produced a phenotype that was only clinically identified a few months after birth. The reduced aminoprocollagen peptidase activity observed *in vitro* in fibroblasts of cases 1 and 2 who do not have an identifiable mutation in the *ADAMTS2* and *14* genes suggests that mutations in other genes can alter the processing of procollagen molecules and result in a clinical phenotype with similarities to the phenotype of EDS type VIIC.

Materials and Methods

Cell culture

Dermal fibroblasts were grown from skin biopsies and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% dialyzed and decomplemented fetal calf serum (FCS) and 50 µg per mL ascorbic acid. The ^{14}C-proline protein labeling, purification of labeled procollagen, and electrophoretic procedures were as described previously (Nuytinck *et al*, 1996).

DNA amplification and sequencing

Total RNA and genomic DNA were purified from fibroblasts in culture. cDNA was synthesized using random primers (9-mers) and Superscript II reverse transcriptase (Invitrogen, Merelbeke, Belgium). Overlapping RT-PCR products were amplified using Taq DNA polymerase (Takara, Bio-Whittaker Europe, Verviers, Belgium) and five different pairs of primers (product 1, 518 bp: 5'-CGCTTGGTGTCCCACGTGGTGT-3' and 5'-CGGCTGAGGCTGTCCAGGCTGT-3'; product 2, 1034 bp: 5'-TTTGGCCGAGACCTGCACCTGC-3' and 5'-AGGTAGCGGCT-CAGCTCCTGCT-3'; product 3, 1110 bp: 5'-GCATGCAAGGC-TATGCTCCTGTCA-3' and 5'-GGATCTCAAACATCTTGATGTAACC-A-3'; product 4, 1149 bp: 5'-CTACAAGGACGCCTTCAGCCTCT-3' and 5'-TGGCAGTGGCCCTTTGACGAGAT-3'; product 5, 1124 bp: 5'-ACAACACCACCCGCTCCGTGCA-3' and 5'-CTGCTTAGCAAC-TTGGGGCCTATT-3'). Amplifications were performed using a 35 cycles program (94°C for 30 s, 66°C for 30 s, and 72°C for 90 sec) in an automated thermal cycler (GeneAmp PCR system 9600, Ap-plera Belgium, Lennik, Belgium). After electrophoresis on agarose gel, the size of the products was evaluated and the DNA was purified from excised agarose plug. The purified DNA products were sequenced as previously described (Colige *et al*, 1999). For amplification of genomic DNA, pairs of primers were designed, allowing the amplification of all the exon/intron junctions of the 22-exon gene. Sequence of the primers and PCR conditions used for data shown in Figs 2 and 3 are available upon request (acolige@ulg.ac.be).

Electron microscopy

Mouse and human skin biopsies were fixed for 60 min at room temperature in 2.5% glutaraldehyde in 0.1 Sörensen's buffer (pH 7.4), post-fixed for 30 min in 0.1% osmium tetroxide in Sörensen's buffer, dehydrated in a series of ethanol concentrations and embedded in epoxy resin (Epon 812, Fluka). Ultrathin sections were stained with uranyl acetate and lead citrate before being examined using a Jeol electron microscope CX100 II at 60 kV or a Philips EM 400 at 80 kV. For P8, the skin biopsy was initially processed for normal histology, then deparaffinized by xylol, refixed in glutaraldehyde in 0.1 M Sörensen's buffer for at least one day and then treated as described above.

Clinical descriptions

All clinical investigations have been conducted according to the local Ethics Committee recommendations and the Declaration of Helsinki principles. Five patients were included in this study. Written informed consent was obtained from the parents of all patients and from the control individuals. Two of them, referred as P7 and P8, had typical hallmarks of EDS type VIIC (Beighton *et al*, 1998). The others, referred as cases 1-3, had only some of the EDS type VIIC symptoms, potentially indicative of a mild defect of the ADAMTS-2 function.

Figure 6. *Frontal view of P7 and P8. (A) P7 at the age of 3 y and (B) P8 at the age of 4 y.*
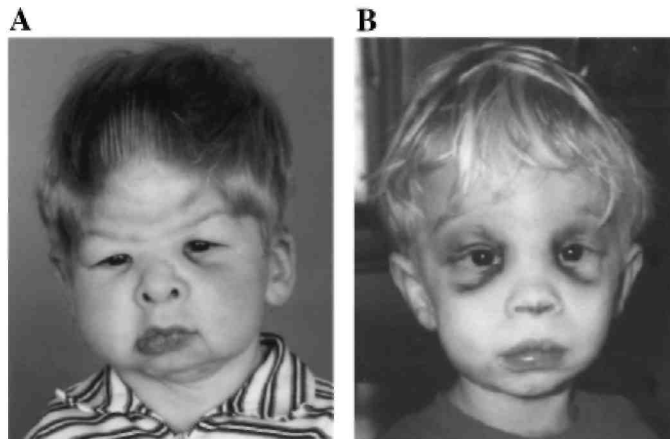
Patient 7 (Figure *6A*) has a healthy sister and his parents, of Dutch Caucasian origin, are healthy and not reported to be related. His mother had four spontaneous abortions. Chromosome analysis was normal in both parents. He was born, at 38 + 4/7 weeks of gestation, by caesarian section because of his large size (3900 g). During delivery, the short umbilical cord (18 cm) ruptured. At birth, he had generalized edema, which gave the face a coarse appearance with swollen eyelids which could not be opened initially. Other craniofacial features included short forehead with hypertrichosis, flat supraorbital ridges, a broad nasal root, wide nares, a smooth philtrum, a large mouth, a thin upper lip, and micrognathia. The large size of the fontanels and the wide cranial sutures were striking. The arms and legs were short. The hands were short and stubby with camptodactyly of the third and fourth fingers bilaterally. When the edema had resolved, sagging skinfolds appeared under the jaw reminiscent of cutis laxa. The skin was very soft and only slowly returned into position after stretching. Psychomotor development was normal. Joint hypermobility was noted at one year of age. He bruised easily as he became more active. He had a fragile skin that tore easily. Wound healing and scar formation were normal. At the current age 7 y, the skeletal growth is normal except for short hands and feet. The main clinical features are still the bleeding tendency, the extensive bruising, gingival hyperplasia, mild joint hypermobility, fragile skin, and blue sclera. Dental features such as hypodontia were observed and will be described further elsewhere (Malfait *et al*, in press).

Patient 8 (Figure *6B*) was born prematurely after 34 weeks of gestation by cesarian section due to breech presentation and polyhydramnios. He was the second child of healthy, unrelated parents. At birth, the only abnormality noted was a large anterior fontanel (3 x 3 cm). Delay in psychomotor development became evident over the ensuing months. There was increased bulging of the anterior fontanel, eyelid edema, umbilical hernia, and bleeding tendency. During three hospital admissions between 6 and 10 months of age, various diagnostic procedures were performed. Normal or irrelevant findings were obtained by ophthalmologic examination, urinary screening for organic acids, visual evoked potentials, and electroencephalograph. The karyotype was normal. Cerebral MRI scan showed an Arnold-Chiari malformation and turricephaly. Repeated coagulation analyses demonstrated a low level of von Willebrand factor, in keeping with a mild form of von Willebrand disease type I. Bone development was delayed. In the second year of life, clinical observations revealed that the skin was very soft and stretchable and that minimal mechanical trauma cause hematomas, wounds, and displacement of joints. The diagnosis of EDS type VIIC was proposed on the basis of the characteristic ultra-structural appearance of collagen fibrils. There was no myopia, osteopenia, or hip dysplasia. The early severe growth retardation resolved progressively. At current age five years, his weight (14 kg) and height (101 cm) are

on the third percentile and his head circumference (51 cm) on the 25th percentile. Oro-dental features such as irregular occlusal morphology and enamel attrition were diagnosed (Malfait *et al,* in press). Recently, he suffered a ruptured urinary bladder diverticulum with urinary ascites. Complications occurred during the operation because of the extreme fragility of the tissues. The main current clinical features are still the bleeding tendency, the extensive bruising, mainly of the buccal mucosa, gingival hyperplasia, hypermobile joints, fragile skin, and blue sclera.

Cases 1-3 Case 1 and 2 were born prematurely at 34 and 32 wk of gestation. They had facial dysmorphisms and a large umbilical hernia. Skin and mucosal fragility, and a tendency to develop hematomas were apparent from the early years of life. Wound healing resulted in atrophic scars. They suffered from a non-traumatic bladder rupture at the age of 5 and 4 y, respectively. Based upon biochemical data and clinical examinations, a diagnosis of EDS type I, type IV, and type VI could be excluded. Normal serum levels of copper and ceruloplasmin excluded the diagnosis of occipital horn syndrome (previously classified as EDS type IX). Case 3 had clinical similarities to cases 1 and 2 such as facial dysmorphisms, skin fragility, presence of atrophic scars, umbilical hernia and a large anterior fontanel which closed with delay. In addition, extreme ligamentous laxity, joint hypermobility, and abnormal palatal formation, with an extra ridge of gingival tissue bilaterally in the molar region, were noted.

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