

Marshall Syndrome Associated with a Splicing Defect at the *COL11A1* Locus

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Summary

Marshall syndrome is a rare, autosomal dominant skeletal dysplasia that is phenotypically similar to the more common disorder Stickler syndrome. For a large kindred with Marshall syndrome, we demonstrate a splice-donor-site mutation in the *COL11A1* gene that cosegregates with the phenotype. The G⁺→A transition causes in-frame skipping of a 54-bp exon and deletes amino acids 726–743 from the major triple-helical domain of the $\alpha 1(\text{XI})$ collagen polypeptide. The data support the hypothesis that the $\alpha 1(\text{XI})$ collagen polypeptide has an important role in skeletal morphogenesis that extends beyond its contribution to structural integrity of the cartilage extracellular matrix. Our results also demonstrate allelism of Marshall syndrome with the subset of Stickler syndrome families associated with *COL11A1* mutations.

Introduction

Craniofacial morphogenesis is a complex process in which unique bony structures are formed around the developing brain (Sperber and Machin 1994; Johnston and Bronsky 1995; Schilling 1997). The recent elucidation of the molecular basis of several osteochondrodysplasias has laid the foundation for a better understanding of the embryologic development of the skull. Mutations have been identified in genes encoding growth-factor receptors, transcription factors, and structural proteins. Examples include defects of the fibroblast growth factor receptor (FGFR) gene family, in the chondrodysplasias (reviewed by Gorlin 1997); defects of

FGFR3 and *MSX2*, in isolated craniosynostoses (Ma et al. 1996; Muenke et al. 1997); defects of *TWIST* (Howard et al. 1997), *CBFA1* (Lee et al. 1997), and *GLI3* (Vortkamp et al. 1991); and defects of type II collagen, in the spondyloepiphyseal dysplasia congenita spectrum (Spranger et al. 1994).

Marshall syndrome (MIM 154780) is an autosomal dominant craniofacial disorder characterized by hypoplasia of the maxilla, nasal bones, and frontal sinuses, as well as calvarial thickening, myopia, early-onset cataracts, and sensorineural hearing loss (Marshall 1958). Some of these clinical features are shared by individuals with Stickler syndrome (hereditary arthro-ophthalmopathy), leading to the suggestion that the conditions may be allelic (Cohen 1974). Stickler syndrome exhibits genetic heterogeneity, and mutations in the genes encoding all three α -chains of type XI collagen (*COL11A1*, *COL11A2*, and *COL2A1*) have been reported (Spranger et al. 1994; Vikkula et al. 1995; Richards et al. 1996). Using a candidate-gene approach, we have excluded linkage of the Marshall syndrome phenotype to *COL11A2* and *COL2A1* and have established linkage to the *COL11A1* locus. We also report a splice-donor-site mutation in *COL11A1* that cosegregates with the clinical phenotype in a large family with Marshall syndrome.

Subjects and Methods

Analysis of the Marshall Syndrome Family

Members of the study family were ascertained at the University of Michigan Medical Center by a clinical geneticist, an ophthalmologist, and an otolaryngologist. After approval by the University of Michigan Institutional Review Board, informed consent was obtained from each study participant.

DNA was extracted from peripheral blood leukocytes by use of the PureGene DNA isolation kit (Gentra Systems). Individuals were typed for the following loci: *COL2A1* (Berg and Olaisen 1993); *COL11A2* (Vikkula

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et al. 1995); *D1S429*, *D1S495*, *D1S239*, *D1S248*, *D6S105*, and *D6S276* (Dib et al. 1996); and *D18S1001* and *D18S1002* (Griffith et al. 1996). For detection of microsatellite-marker products, one PCR primer (Research Genetics) was 5' end-labeled with γ -[³²P]ATP. Amplifications were performed by use of *Taq* polymerase (Boehringer Mannheim), with an initial denaturation for 3 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C, in an MJ Research PTC-100 thermal cycler. Amplification products were visualized by autoradiography, after electrophoresis on 6% polyacrylamide gels containing 7M urea.

Mapping of COL11A1 and Identification of Closely Linked Markers

A human genomic phage clone was isolated by library screening with a 2.5-kb *COL11A1* cDNA probe (Bernard et al. 1988). Sequence analysis identified a (CA)_n simple-sequence repeat that can be amplified from human genomic DNA with primers 5'-TTT ATG TCT TGT TCT GTT TGA-3' (sense) and 5'-CTT AAT GGA GAA TTA GAA TGG-3' (antisense). Thirty-two informative CEPH pedigrees were analyzed. Pairwise linkage analyses were performed between *COL11A1* and loci on human chromosome 1, from the CEPH database (www.cephb.fr), by use of the CLODSCORE portion of the LINKAGE programs (version 5.10), kindly supplied by Dr. Jurg Ott (Lathrop et al. 1984). The sex-specific recombination rates for males (θ_m) and those for females (θ_f) were set to be equal for the analyses. Locus-order calculations were done by use of CILINK, and final order testing was done by comparison of the location scores for all the loci in their predicted order and then testing of the inverted orders, also by use of CILINK.

Reverse-Transcription (RT) PCR

Trizol reagent (Gibco BRL) was used to extract total RNA from primary cultures of dermal fibroblasts derived from individual II-1. First-strand cDNA products were prepared from 1 μ g total RNA separately with oligo(dT) primer or random hexamer primers, by use of the SuperScript system (Gibco BRL). Overlapping cDNA fragments were generated by nested PCR amplification of the resulting RT products. The first-round PCR primers, listed in table 1, were the following sense ("F")/antisense ("R") pairs: 1F/13R, 12F/5R, 17F/19R, 14F/20R, 18F/7R, 6F/5104R, and 7F/9R. Two microliters of RT products were amplified in 50 μ l reaction volumes with *Taq* polymerase. Enzyme was added after initial denaturation for 4 min at 95°C and cooling to 80°C for 1 min, followed by 29 cycles of 40 s at 95°C; 40 s at 52°C (primer pair 1F/13R), 58°C (primer pairs 12F/5R, 14F/20R, 6F/5104R, and 7F/9R), or 64°C (primer pairs

Table 1

Primers Complementary to the COL11A1 Coding Sequence

Primer(s)	Nucleotides
1F	1–20
11F	129–148
2F	688–707
12F, 12R	1093–1113
3F, 3R	1400–1419
13R	1716–1735
17F	1746–1765
4F, 4R	2011–2030
2161F	2161–2180
14F	2317–2336
18F, 18R	2341–2360
5F, 5R	2711–2731
2956R	2937–2956
19F, 19R	3081–3100
16F, 16R	3255–3275
6F, 6R	3540–3560
20F, 20R	3852–3871
3928F	3928–3947
4027R	4007–4027
7F, 7R	4180–4199
8F, 8R	4831–4851
8R2	4840–4860
5104R	5084–5104
9R	5543–5563

NOTE.—Nucleotide numbers correspond to the cDNA sequence (GenBank J04177 and J05407). F = sense primer; and R = antisense primer.

17F/19R and 18F/7R); 3 min at 72°C; and a final extension of 10 min at 72°C. Nested second-round amplifications were performed similarly, to yield smaller fragments internal to each of the first-round products. Two microliters of first-round products were added as substrate for each 50- μ l nested PCR reaction. The cycling parameters, with the corresponding primer pairs indicated parenthetically, included initial heating to 95°C for 4 min; 40 s at 95°C; 40 s at 54°C (2F/3R), 58°C (11F/12R, 12F/13R, 3F/4R, 4F/5R, 2161F/2956R, and 7F/5104R), 62°C (5F/6R, 19F/20R, 16F/7R, 20F/8R, 20F/8R2, and 8F/9R), or 66°C (17F/18R and 18F/16R); and 1 min 20 s at 72°C, for 35 cycles, with an extension of 10 min at 72°C.

Mutation Detection in cDNA by Conformation-Sensitive Gel Electrophoresis

Second-round RT-PCR products were analyzed directly by conformation-sensitive gel electrophoresis, as described elsewhere (Ganguly et al. 1993; Raman et al. 1997). PCR products were digested with the restriction enzymes *Apa*I, *Eco*RI, *Fok*I, *Hha*I, *Hinf*I, *Hpa*II, *Rsa*I, or *Sty*I, to produce 200–600-bp fragments for analysis. The *Sty*I-digestion fragments of the 20F/8R2 PCR product derived from individual II-1 were excised from an

agarose gel and were purified with Qiaex II (Qiagen), for sequencing. Cycle sequencing was performed by use of ThermoSequenase (Amersham), according to the manufacturer's instructions.

Mutation Detection in Genomic DNA

Primers 3928F and 4027R (table 1) were designed from the cDNA sequence flanking the downstream boundary of the 54-bp deleted segment and were used to amplify genomic DNA from individual II-1. The 1-kb amplification product was excised from a 1.5% agarose gel and was purified with Qiaex II for cycle sequencing. Antisense primers COL INT R1 (5'-TTA ATG AGT TGG GAA GGG AAA G-3') and COL INT R2 (5'-TTC GAA CCA CAG CTA AGA GTT G-3') were designed from the downstream intron sequences (nucleotide positions +101 to +122 and +74 to +95, respectively), for nested PCR with sense primer 3928F, to amplify the exon/intron boundary from each family member. The 3928F/COL INT R2 amplification products were analyzed directly by cycle sequencing.

Results

Marshall Syndrome Phenotype

The study family included nine living affected individuals, from three generations, with characteristic features of Marshall syndrome (Marshall 1958; Ayme and Preus 1984). These features included a markedly short nose with flat root, depression of the nasal bridge, anteverted nostrils, and retracted midface (fig. 1A and 1B). Four of the affected individuals had cleft palate and recurrent otitis media, which also were associated with Pierre Robin sequence, in individual III-2. Four other affected individuals had bifid uvula. No dental anomalies were observed. Affected individuals had myopia and cataracts, which became apparent during the 1st decade of life and were located in subcapsular, cortical, nuclear, zonular, or anterior axial embryonic sites. Fluid vitreous humor was present in all affected individuals undergoing cataract surgery. Sensorineural hearing loss was noted as early as 3 years of age, with gradual progression to moderate or severe levels by late adulthood. Other features included short stature, relative to unaffected family members, and stocky build. Early-onset (i.e., 4th or 5th decade) symptomatic osteoarthritis affecting the knees and lumbosacral spine and mild hypotrichosis and hypohidrosis were present in some affected family members. Individual III-2 had mild platyspondylia, and two individuals had bilateral upper-extremity clinodactyly of the fourth and fifth digits. Radiologic findings included severe hypoplasia of the maxilla, nasal bones, and frontal sinuses (fig. 1C), calvarial

thickening and intracranial calcifications (fig. 1D), and narrowed joint spaces, with osteophytic degeneration in the hips (fig. 1E) and knees (fig. 1F).

Linkage Mapping of COL11A1 to Human Chromosome 1p21

Amplification of genomic DNA from 50 normal individuals, by use of PCR primers flanking a (CA)_n repeat in the COL11A1 gene, identified six alleles 154–170 bp in size. Allele frequencies were .06–.22, with an observed heterozygosity of .70. The genotypes of CEPH pedigree members 1331-01 and 1331-02 were 154/170 and 154/162, respectively. COL11A1 showed no recombination with markers D1S429, D1S495, D1S239, and D1S248 (LOD score >11.74). The most likely locus order is AMY2B-2 cM-(COL11A1, D1S429, D1S495, D1S239, D1S248)-5 cM-D1S221, with odds of 7×10^{-6} for the next most likely order. These data confirm the previous mapping of COL11A1 to human chromosome 1p21 by FISH analysis (Henry et al. 1988), and is consistent with the linkage of Amy2 and COL11a1 on mouse chromosome 3 (Li et al. 1995).

Linkage and Haplotype Analysis of the Marshall Syndrome Family

We tested four candidate genes for linkage to Marshall syndrome: COL2A1, COL11A1, COL11A2, and the human orthologue of the mouse craniofacial mutant Tg9257. At least one obligatory recombination was observed with the COL2A1 gene VNTR (Berg and Olaisen 1993), the Tg9257 markers D18S1001 and D18S1002 (Griffith et al. 1996), and the COL11A2 flanking markers D6S105 and D6S276 (data not shown). In contrast, there was no recombination between the COL11A1 marker D1S248 and Marshall syndrome, in 12 informative meioses (fig. 2). D1S206, D1S239, D1S429, and D1S495 also segregated concordantly with the phenotype. Thus, the Marshall syndrome locus is closely linked to COL11A1.

Abnormal COL11A1 Transcript in Affected Individuals

Overlapping cDNA fragments corresponding to the COL11A1 open reading frame were prepared by RT-PCR using dermal fibroblast RNA from affected individual II-1 (fig. 2) and from unaffected controls. Conformation-sensitive gel electrophoresis of RT-PCR products spanning the open reading frame detected a single difference between the affected individual and the controls: an extra band in the 1.1-kb RT-PCR product obtained with primers 20F and 8R2 (table 1). Digestion of this 1.1-kb cDNA fragment with FokI generated a unique 513-bp fragment in the sample from the affected individual (fig. 3). Comparison of the nucleotide sequence of the RT-PCR product from the patient and that



Figure 1 Marshall syndrome phenotype of individual II-1. *A* and *B*, Photographs showing the short nose with flat root, sunken nasal bridge, retruded midface, and mild orbital hypertelorism. *C*, Cranial radiograph showing hypoplastic nasal bones (*arrowheads*). *D*, Cranial computed tomography showing intracranial calcifications (*arrows*). *E* and *F*, Radiographs of the hip (*E*) and knee (*F*), showing narrowing of joint spaces and osteophytic changes.

from the controls revealed an internal 54-bp in-frame deletion in the patient that corresponds to nucleotides 3924–3977 of the *COL11A1* cDNA (numbered according to GenBank accession nos. J04177 and J05407 [http://www.ncbi.nlm.nih.gov]; Bernard et al. 1988; Yoshioka and Ramirez 1990). The deleted sequence encodes 18 amino acids, corresponding to residues 726–743 of the triple-helical domain of the mature $\alpha 1(XI)$ polypeptide (Bernard et al. 1988).

Mutation Detection in Genomic DNA

To determine the molecular basis for the 54-bp deletion in the *COL11A1* cDNA, genomic DNA was am-

plified with cDNA primers 3928F and 4027R (table 1), flanking the 3' end of the deleted sequence. The 1-kb genomic amplification product from affected individual II-1 was sequenced (GenBank accession no. AF017809). Comparison with the cDNA demonstrated the presence of two exons and one intron in the amplified fragment. Downstream of the first exon, heterozygosity for a G→A transition at the +1 position of the splice-donor site was detected (fig. 4, *left panel*). This mutation changes the splice donor in the wild-type allele from the consensus-site sequence GTAAGT to the nonconsensus sequence ATAAGT (fig. 5A). The remaining sequence was identical in the two alleles. Genomic DNA from the other

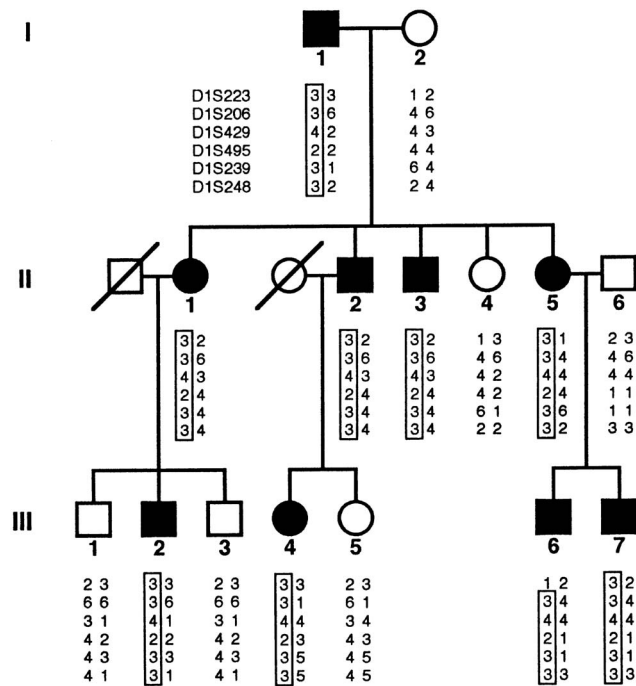


Figure 2 Pedigree showing linkage of Marshall syndrome phenotype with *COL11A1* markers from chromosome 1p21.

family members was tested for the splice-site mutation by sequencing the amplified PCR product. Cosegregation of the mutation with the disorder is demonstrated in figure 4 (*right panel*). Using MLINK (Lathrop et al. 1984), with $q = .0001$, we obtained a LOD score of 3.31 (at $\theta = 0$), between the disease phenotype and the mutant allele.

Discussion

Exon skipping due to the splice-site mutation in this Marshall syndrome kindred is predicted to cause an 18-amino-acid deletion in the major triple-helical domain of the mature $\alpha 1(XI)$ polypeptide (fig. 5B). Although determination of the genomic organization of *COL11A1* is incomplete, the position of the 54-bp sequence that is deleted from the patient cDNA corresponds precisely to exon 49 in the closely related fibrillar collagen gene *COL5A1* (Takahara et al. 1995). Mutations of the consensus G at the +1 position of the splice-donor site frequently are associated with exon skipping (Horowitz and Krainer 1994). Similar exon-skipping mutations have been described in other fibrillar collagen genes (Kuivaniemi et al. 1997), including *COL11A2* (Vikkula et al. 1995) and *COL2A1* (Tiller et al. 1995). It has been proposed that the resulting mutant polypeptides associate with other α -chains via the intact C-propeptide domain to form heterotrimers, but fail to form

a normal collagen triple helix. The incomplete formation of the triple helix may exert a dominant-negative effect on collagen synthesis, secretion, fibril assembly, or turnover (Tilstra and Byers 1994). The dominant mutation in this Marshall syndrome kindred may act via this mechanism.

The genetic relationship between Marshall syndrome and Stickler syndrome has been debated extensively. The kindred described here exhibits several clinical features that serve to support its classification as a Marshall syndrome kindred: (1) persistence of the flat nasal root and depressed nasal bridge through adulthood; (2) severe hypoplasia of the nasal bones and frontal sinuses; (3) calvarial thickening; and (4) intracranial calcifications (Marshall 1958; Ayme and Preus 1984; Shanske et al. 1997). In addition, the short stocky build and round face observed in affected members of this kindred are most consistent with Marshall syndrome.

Mutations in *COL2A1*, *COL11A1*, and *COL11A2* have been reported in kindreds with Stickler syndrome (Spranger et al. 1994; Vikkula et al. 1995; Richards et al. 1996), indicating that some cases of Stickler syndrome are allelic with Marshall syndrome. Clinical photographs and cranial radiographs of affected individuals with Stickler syndrome due to mutation at the *COL11A1* locus (Richards et al. 1996) or linked to the *COL11A1* locus (Sirka-Osada et al. 1996) have not been published, and it is possible that their craniofacial phenotype may be closer to that of Marshall syndrome. Molecular characterization of additional families with both syndromes will be required to clarify their genetic relationship.

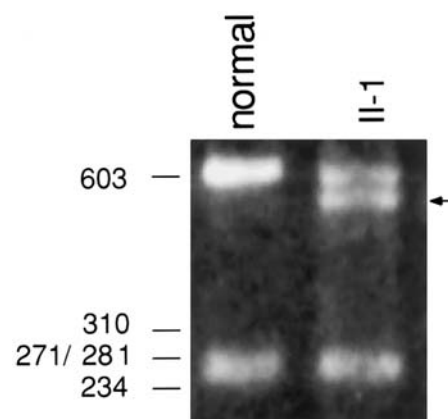


Figure 3 Abnormal *COL11A1* transcript in Marshall syndrome. The RT-PCR products amplified with primers 20F and 8R2 (table 1), from affected individual II-1 and from an unaffected control, were digested with *FokI* and were separated by agarose-gel electrophoresis. A novel restriction fragment present only in the affected individual is indicated by an arrow. The relative mobilities of molecular-weight markers (Φ X174/*HaeIII*) are shown to the left.

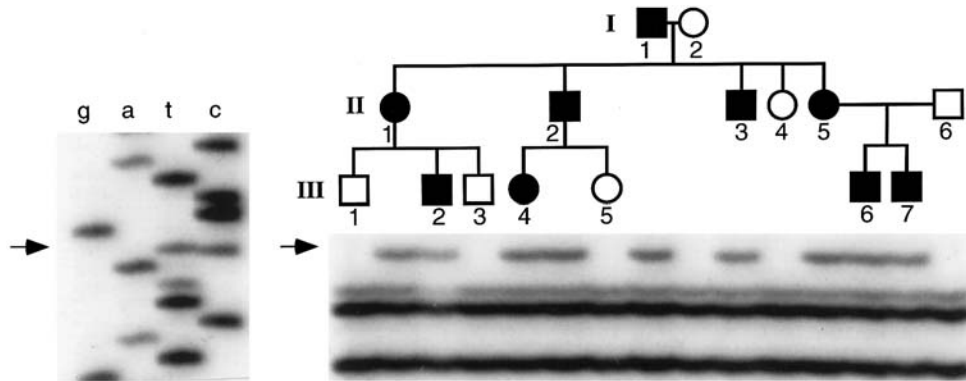


Figure 4 Genomic *COL11A1* mutation. *Left*, Sequence of genomic DNA from noncoding strand of affected individual II-1, showing heterozygosity for a C→T transition at position +1 of the downstream intron. *Right*, ddTTP–chain termination products (corresponding to lane t in left panel) from sequence analysis of kindred members, showing cosegregation of the C→T transition with the phenotype.

A phenotypic spectrum has been described for mutations in several collagen genes (Spranger et al. 1994; Tilstra and Byers 1994) and in other genes expressed in connective tissue (Oldridge et al. 1997). Genotype/phenotype correlations among allelic mutations can provide insight into the functions of various protein domains. If Marshall syndrome and Stickler syndrome are allelic, we predict that *COL11A1* mutations associated with the Marshall syndrome phenotype will be more disruptive to protein structure than those causing Stickler syndrome.

The distinct craniofacial features observed in Marshall syndrome suggest that, during development, the $\alpha 1(XI)$ polypeptide has a unique role distinct from that of the other collagen polypeptides with which it forms heterotrimers. This hypothesis is consistent with the differ-

ences in expression patterns of these genes (Mayne et al. 1993; Lui et al. 1995; Yoshioka et al. 1995a; Tsumaki et al. 1996), including the specific expression of *COL11A1* in developing nasal structures (Yoshioka et al. 1995b). These genes also differ in tissue-specific alternative splicing (Oxford et al. 1995; Yoshioka et al. 1995a; Zhidkova et al. 1995) and in their contribution to heterotrimer assembly and heterotypic fibril formation, including the association of $\alpha 1(XI)$ subunits with $\alpha 2(V)$ subunits to form heterotrimeric V/XI molecules in noncartilaginous tissues such as bone and vitreous humor (Niyibizi and Eyre 1989; Mayne et al. 1993). Differences in the vitreoretinal phenotype among Stickler syndrome kindreds that are linked or unlinked to the *COL2A1* locus have been observed (Snead et al. 1994). In addition, unlike Stickler syndrome patients with mutations in *COL2A1* or *COL11A1*, there is no ocular involvement in three Stickler syndrome families with mutations in *COL11A2* (Vikkula et al. 1995; Sirko-Osadasa et al., in press).

In addition to their well-documented role in establishing and maintaining structural integrity of connective tissues, new roles for the fibrillar collagens are becoming evident. In the mouse mutant chondrodysplasia (*cho* gene), a null mutation of *COL11A1* produces an autosomal recessive lethal phenotype with nasomaxillary hypoplasia and disturbances in the morphogenesis of endochondral growth plates (Li et al. 1995). The phenotype of the Marshall syndrome kindred described here demonstrates a similar role for *COL11A1* in human craniofacial development. The related defects in mouse mutant Tg9257 suggests that its orthologous gene on human chromosome 18q11 also may contribute to the spectrum of human craniofacial disorders (Griffith et al. 1996). Continued biochemical and molecular studies of human families and mouse models will further our un-

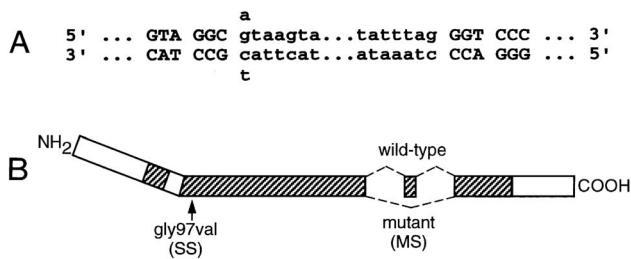


Figure 5 A, Splice-donor-site mutation in *COL11A1*. The C→T transition corresponds to a G→A transition in the coding strand, altering the canonical GT dinucleotide of the consensus splice-donor-site sequence at the exon (uppercase letters)–intron (lowercase letters) boundary. B, Mutations in the $\alpha 1(XI)$ collagen polypeptide. The schematic diagram of the $\alpha 1(XI)$ polypeptide illustrates the sites of the 18–amino-acid deletion in the Marshall syndrome kindred and the Gly97Val missense mutation in the Stickler syndrome kindred reported by Richards et al. (1996). Hatched areas indicate domains containing Gly-X-Y repeat motifs that assemble into triple helices with other collagen subunits.

derstanding of the complex processes involved in cephalogenesis.

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