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POLYMERASE CHAIN REACTION - AMPLIFICATION OF THE CODING SEQUENCE OF THE TYPE X COLLAGEN GENE FROM GENOMIC DNA AND IDENTIFICATION OF A POLYMORPHISM THAT CHANGES GLY TO ARG AT POSITION 545 BY SINGLE-STRAND CONFORMATION POLYMORPHISM ANALYSIS

Rita M. Dharmavaram, Minzhong Peng, Rendall R. Strawbridge and Sergio A. Jimenez*

Division of Rheumatology, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Bluemle Life Sciences Building, Suite 509, 233 South 10th Street, Philadelphia, PA 19107

Received July 7, 1992

Type X collagen is a specific product of hypertrophic growth plate chondrocytes and it has been suggested that mutations in the corresponding gene (COL10A1) may be responsible for certain heritable disorders affecting growth plate cartilage such as the epiphyseal dysplasias. We have amplified the coding region of COL10A1 employing polymerase chain reaction (PCR) of genomic DNA. Single-strand conformation polymorphism (SSCP) analysis of PCR products followed by direct sequencing identified a G to C transition that results in a Gly to Arg substitution at position 545 of the polypeptide chain. The sequence variation was confirmed by restriction enzyme analysis with BsaJ 1. Analysis of a family with multiple epiphyseal dysplasia ruled out this sequence change as a cause of the disease. This is the first report showing application of SSCP for detection of a sequence variant in COL10A1.

Type X collagen is a short chain non-fibril forming collagen synthesized exclusively by hypertrophic chondrocytes of the growth plate (1). It is expressed almost exclusively in growth plate cartilage and it has been suggested that mutations in the gene coding for this collagen (COL10A1) may be responsible for certain heritable disorders of cartilage such as the epiphyseal dysplasias. The entire coding sequence of COL10A1 has recently been reported and the gene has been localized to 6q21 - 6q22.3 (2).

As a first step towards detecting mutations in COL10A1 we have designed primers that allowed us to amplify the coding sequence of this gene from genomic DNA utilizing PCR. The amplified PCR products were subjected to SSCP analysis (3). The mobility shift of PCR products on

*To whom correspondence should be addressed.

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SSCP gels was utilized for rapid detection of sequence variations in COL10A1 type X collagen gene. Direct sequencing of the PCR products displaying altered mobility on SSCP gels allowed the identification of a G to C transition at nucleotide 1633 of the gene resulting in a Gly to Arg substitution at position 545 of the polypeptide chain. The nucleotide sequence variation was predicted to result in the loss of a BsaJ 1 restriction enzyme cleavage site which was confirmed by BsaJ 1 digestion of the PCR product. The allele with the sequence variant occured in 15% of the population examined. Analysis of affected and unaffected individuals of a family with multiple epiphyseal dysplasia of the Fairbank type (4) ruled out this sequence change as a cause of the disease.

METHODS

Genomic DNA was isolated as described previously (5). The primer sets shown in Table 1 were utilized for PCR. Conditions for all PCR amplifications were as follows: initial denaturation at 94°C for 10 sec followed by 94°C for 7 sec, 54°C for 20 sec, 72°C for 1 min 10 sec for 35 cycles and a final extension at 72°C for 1 min on a GeneAmp PCR System 9600 (Cetus). The first symmetric PCR was carried out with 100 pmol of each of the primer sets. DNA sequencing was performed with products of two successive asymmetric PCR amplifications. The first asymmetric PCR was carried out using 20 pmol of X-9 and 5 pmol of X-10 and 1 μl of product from the first symmetric PCR. The second asymmetric PCR was carried out using 60 pmol of X-9 and 0.8 pmol of X-10 and 1 μl of product from the first asymmetric PCR. The single stranded PCR products were sequenced using X-11 as the sequencing primer by the Sequenase 2.0 kit (United States Biochemical).

SSCP analysis was performed by the method of Orita *et al* (3) except that instead of acrylamide the mutation detection enhancement gel system from AT Biochem was used. The gels were electrophoresed at room temperature.

RESULTS AND DISCUSSION

As shown in Figure 1A we amplified 1940 bp of the coding sequence of COL10A1. The oligonucleotide primers employed for this purpose are listed in Table 1. The position and length of the PCR fragments shown in Figure 1A are indicated in Figure 1B. Sequencing of these PCR fragments indicated that their sequence was identical to that of corresponding regions coding for the type X collagen reported previously (2).

SSCP analysis of PCR fragment 5 obtained by amplification of genomic DNA with primers X-9 and X-10 from thirty-eight individuals revealed two additional bands in eleven individuals. The presence of bands with normal mobility indicated that the eleven individuals were

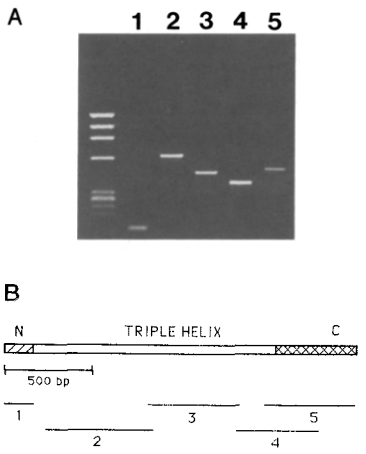

Fig. 1. Amplification of the coding sequence of the type X collagen gene from genomic DNA. Amplification was carried out as described in Methods and one-tenth of the PCR product was electrophoresed on a 1% (W/V) agarose gel and stained with ethidium bromide.

A. Amplification was carried out using the primer sets shown in Table 1. øX174 digested with Hae III was used as a marker. Amplifications were carried out with the following primers:
Lane 1 X-1/X-2 Lane 2 X-3/X-4 Lane 3 X-5/X-6 Lane 4 X-7/X-8
Lane 5 X-9/X-10.

B. Map of the coding sequence of the type X collagen gene indicating the length and position of fragments shown in A.

heterozygotes. Therefore, the frequency of the allele with the sequence variant was 15% in the population examined. The pattern of migration of PCR products shown in Figure 2 is representative of that obtained from

Table 1. POSITION AND SEQUENCE OF OLIGONUCLEOTIDE PRIMERS[a]

Primer number	Primer sequence (5′ to 3′)	Location	Expected product size (bp)
X-1	ATG CTG CCA CAA ATA CCC	1-18	133
X-2	AAT GAA GAA CTG TGT CTT	132-115	
X-3	CCA CCA GGA AAA CCA GGC	235-252	615
X-4	GAG ACC TTT TGT TCC TGG AAT	849-829	
X-5	ATT CCA GGA ACA AAA GGT CTC	829-849	525
X-6	TCT AGT ACC TGG TAT TCC AGG	1353-1333	
X-7	CCT GGA ATA CCA GGT ACT AGA	1333-1353	480
X-8	ATG CAC GTG GTA TGA AAA ATA	1812-1792	
X-9	CAC TCT GGA GAG CCT GGT	1498-1515	546
X-10	TCA CAT TGG AGC CAC TAG	2043-2026	
X-11	CAA AAT TTT ATC AAA TGG	2025-2008	

[a]Based on the nucleotide sequence of COL10A1 from Thomas et al (2).

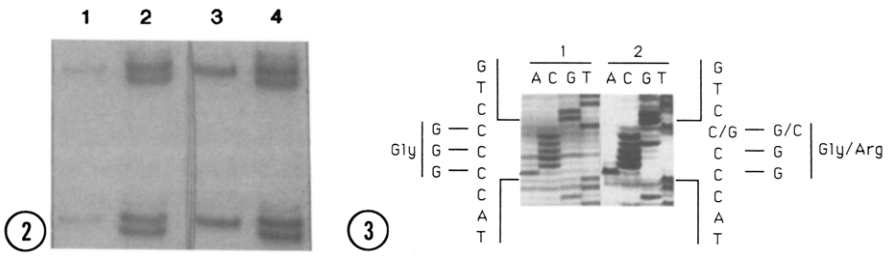

Fig. 2. SSCP analysis of PCR fragment 5 from four different individuals. Note that in two individuals (lanes 1 and 3) only two bands are present, whereas in each of the other two individuals (lanes 2 and 4) two additional bands with faster migration are present.

Fig. 3. Sequencing analysis of PCR fragment 5 showing 1. normal sequence 2. sequence of the G/C heterozygote. Sequencing was carried out as described in methods using primer X-11.

two individuals without the abnormal allele showing a single PCR product (Lanes 1 and 3) and two individuals with the abnormal allele showing additional PCR-amplified bands (Lanes 2 and 4).

To determine the nucleotide change responsible for the altered mobility of PCR products on SSCP gels, the PCR products were directly sequenced with sequencing primer X-11. Sequence analysis indicated that the individuals with the additional DNA bands on SSCP gels were heterozygous at nucleotide position 1633 as shown in Figure 3. The nucleotide transition results in a change of the codon for glycine (GGG) to a codon for arginine (CGG) in one allele. It was predicted that in individuals lacking the variant allele digestion of PCR fragment 5 with the restriction enzyme BsaJ 1 would result in three fragments of 286 bp, 121 bp and 79 bp. Furthermore, in the individuals heterozygous for the G/C transition BsaJ 1 restriction would yield a 200 bp fragment in addition to the three fragments obtained from digestion of the normal allele due to the loss of a BsaJ 1 site in the variant allele. This was in fact observed as shown in Figure 4. Lanes 1 and 2 are BsaJ 1

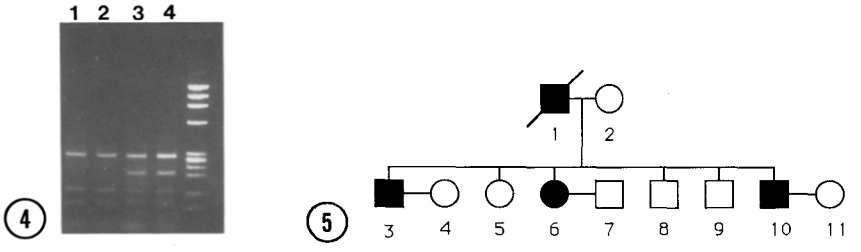

Fig. 4. BsaJ 1 digestion of PCR fragment 5. øX174 digested with Hae III was used as a marker. Lanes 1 and 2. Digestion of PCR fragments shown in lanes 1 and 3 of Figure 2, respectively. Lanes 3 and 4 Digestion of PCR fragments shown in lanes 2 and 4 of Figure 2, respectively.

Fig. 5. Partial pedigree of the multiple epiphyseal dysplasia family that was analyzed for the presence of the sequence variant. Filled symbols: Affected individuals. Open symbols: Normal individuals.

digestions of PCR-amplified fragment 5 from individuals without the sequence variant and Lanes 3 and 4 are BsaJ 1 digestions of the same fragment from individuals with the sequence variant.

The inheritance of the sequence variant was examined in a family with the Fairbank form of multiple epiphyseal dysplasia described in detail previously (6). The portion of the family pedigree examined is illustrated in Figure 5. PCR fragment 5 was amplified from genomic DNA isolated from two normal individuals (individuals 2 and 6 in Figure 5) and one affected individual (individual 11 in Figure 5). Digestion of the PCR fragment with BsaJ 1 resulted in the expected cleavage of the normal sequence in the three individuals indicating that none of them had the sequence variant. Therefore, we concluded that this sequence variant is a polymorphism and is not responsible for the multiple epiphyseal dysplasia phenotype in this family.

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