Molecular Analysis of Collagen XVIII Reveals Novel Mutations, Presence of a Third Isoform, and Possible Genetic Heterogeneity in Knobloch Syndrome

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Knobloch syndrome (KS) is a rare disease characterized by severe ocular alterations, including vitreoretinal degeneration associated with retinal detachment and occipital scalp defect. The responsible gene, *COL18A1,* has been mapped to 21q22.3, and, on the basis of the analysis of one family, we have demonstrated that a mutation affecting only one of the three *COL18A1* isoforms causes this phenotype. We report here the results of the screening of both the entire coding region and the exon-intron boundaries of the *COL18A1* gene (which includes 43 exons), in eight unrelated patients with KS. Besides 20 polymorphic changes, we identified 6 different pathogenic changes in both alleles of five unrelated patients with KS (three compound heterozygotes and two homozygotes). All are truncating mutations leading to deficiency of one or all collagen XVIII isoforms and endostatin. We have verified that, in exon 41, the deletion c3514-3515delCT, found in three unrelated alleles, is embedded in different haplotypes, suggesting that this mutation has occurred more than once. In addition, our results provide evidence of nonallelic genetic heterogeneity in KS. We also show that the longest human isoform (NC11-728) is expressed in several tissues (including the human eye) and that lack of either the short variant or all of the collagen XVIII isoforms causes similar phenotypes but that those patients who lack all forms present more-severe ocular alterations. Despite the small sample size, we found low endostatin plasma levels in those patients with mutations leading to deficiency of all isoforms; in addition, it seems that absence of all collagen XVIII isoforms causes predisposition to epilepsy.

Introduction

Knobloch syndrome (KS [MIM 267750]) is an autosomal recessive disorder characterized by high myopia, vitreoretinal degeneration with retinal detachment, macular abnormalities, and occipital encephalocele. Clinical variability is present, but all patients have ocular abnormalities that usually lead to bilateral blindness. The occipital encephalocele is a major clinical feature that has been described in 22 of 24 reported cases (Knobloch and Layer 1971; Czeizel et al. 1992; Seaver et al. 1993; Passos-Bueno et al. 1994; Wilson et al. 1998; Sniderman et al. 2000). Other minor clinical abnormalities—such as lens subluxation, cataracts, hypoplasia of the right lung with anomalous pulmonary return, cardiac dextroversion, flat nasal bridge, midface hypoplasia, bilateral epicanthic folds, generalized hyperextensibility of the joints, unilateral duplicated renal collecting system, and unusual palmar creases—have been observed in single families (Knobloch and Layer 1971; Czeizel et al. 1992; Seaver et al. 1993; Passos-Bueno et al. 1994; Wilson et al. 1998). Recently, a patient with midline frontal-region scalp defect associated with high myopia, vitreoretinal degeneration, and abnormal macular pigmentation had a diagnosis of KS (Sniderman et al. 2000). It is still unclear whether all of these features are part of the clinical spectrum of KS.

KS seems to be a rare condition, with only 24 patients from six unrelated families having been reported to date (Knobloch and Layer 1971; Czeizel et al. 1992; Seaver et al. 1993; Passos-Bueno et al. 1994; Wilson et al. 1998; Sniderman et al. 2000). On the basis of the study of a single large, inbred Brazilian family with 12 individuals affected with KS, we previously mapped the disease gene to 21q22.3 and demonstrated that a homozygous mutation (IVS1-2A→T) at the AG consensus acceptor splice site of *COL18A1* intron 1 causes KS (Sertié et al. 2000).

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The *COL18A1* gene includes 43 exons and is reported to encode two distinct isoforms in humans by use of two promoters, one of which is located upstream from exon 1 and the other of which is located upstream from exon 3. Use of the upstream promoter yields a product that contains exons 1 and 2 but excludes exon 3, whereas the product of the downstream promoter starts in exon 3 (Saarela et al. 1998*b*) (fig. 1). Therefore, these two isoforms differ only at their signal peptides, with variant N-terminal noncollagenous domains ("NC11's," according to the nomenclature outlined by Oh et al. [1994*a,* 1994*b*]) that are 303 (short variant; NC11-303) and 493 (median variant; NC11-493) residues in length. In the mouse orthologue gene, the variant NC11-764, corresponding to a third collagen XVIII isoform, which has not yet been fully characterized in humans, is also transcribed from the promoter in intron 2 but differs from the mouse NC11-517 (comparable to the human NC11-493) by the inclusion of the entire third exon (Oh et al. 1994*a;* Saarela et al. 1998*a,* 1998*b*). Collagen XVIII was shown, by sequence analysis, to consist of a central, interrupted triple-helical domain and to be flanked at the N-terminus (NC11 domain) and the C-terminus (NC1 domain) by larger non–triple-helical, presumably globular structures (Oh et al. 1994*a,* 1994*b;* Rehn and Pihlajaniemi 1994). A 20-kDa proteolytic cleavage product, endostatin, is derived from its C-terminal NC1 domain. Endostatin can inhibit angiogenesis and can decrease endothelial cell migration and proliferation (O'Reilly et al. 1997). It has also been found to have an effect on apoptosis and migration of other cell types, including neurons and renal epithelial cells (Ackley et al. 2001; Lin et al. 2001). The formation of collagen XVIII homotrimers is probably controlled by a 50-residue region within the NC1 domain (Sasaki et al. 1998). This collagen was shown to be expressed in a large number of tissues, to be localized in vessel walls and almost all basement membranes by using antibodies against NC11 domains, and to have some differences in the expression of each isoform in different tissues (Saarela et al. 1998*a,* 1998*b*). The NC11-493 mRNA variant is mainly expressed in fetal and adult liver, whereas the NC11-303 variant was found at the highest levels in the fetal and adult kidney (Saarela et al. 1998*a,* 1998*b*). This short isoform is also expressed in human retina and fetal brain, which are the tissues almost invariably affected in patients with KS (Sertié et al. 2000). In contrast, in human tissues, the expression of the human counterpart of the mouse NC11-764 variant (predicted to be NC11-728) has not been described.

The mutation at the acceptor splice site of *COL18A1* intron 1 (i.e., IVS1-2A→T, identified in the original family that we studied) abolished the function of only the short variant (NC11-303) of collagen XVIII. Therefore, the characterization of *COL18A1* mutations in other patients with KS, including those with atypical phenotypes, will be important to ascertain whether mutations in other regions of the gene cause a similar phenotype, to assess for genetic heterogeneity, and, finally, to better define the spectrum of clinical variability in this syndrome. In the present article, we have also evaluated the expression pattern of the NC11-728 variant (the counterpart for the mouse NC11-764 variant) in several human tissues.

Subjects, Material, and Methods

Subjects

Eight unrelated families with patients who had a diagnosis of KS were referred to our center (Centro de Estudos do Genoma Humano) at the University of São Paulo; four of these families were Brazilian (KS2, KS3, KS8, and KS9), two were North American (KS4 and KS5), one was Hungarian (KS6), and one was of Canadian-Haitian origin (KS7). The clinical description of the last two families has been reported elsewhere (Czeizel et al. 1992; Sniderman et al. 2000), and the six other families are described here for the first time. These patients present occipital encephalocele and the typical ocular alterations described in patients with KS; no other major malformation was observed. Five families (KS2, KS4, KS6, KS8, and KS9) represented familial cases, and the other three (KS3, KS5, and KS7) represented sporadic cases. Consanguinity was observed in three genealogies (KS2, KS8, and KS9).

DNA Extraction

Blood was drawn after written informed consent was obtained, and DNA was extracted using a standard procedure (Miller et al. 1988).

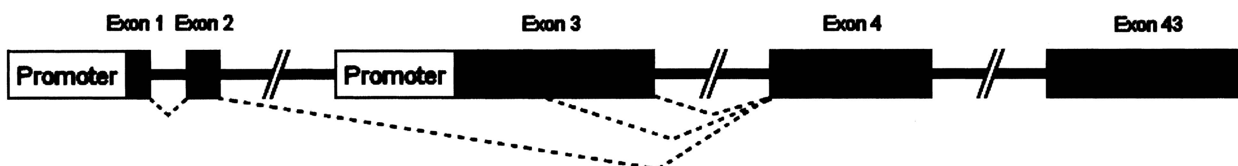

Figure 1 Diagram of the N-terminal region of *COL18A1,* showing the two distinct promoters and the alternate transcription start sites

Mutation Analysis of the COL18A1 *Gene*

We amplified each of the 43 exons of the *COL18A1* gene through PCR in seven of the eight propositi. In one case, the DNA from the patient (KS6) ran out, and we analyzed all the coding sequence and the exon-intron borders of the DNA from his parents, except for exon 1 of the patient's father. The primer-pair sequences used are available in table A (online only). Each exon was screened for mutations through bidirectional sequencing, which was performed with the same primers that were used for PCR amplification and with DyeDeoxy Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the ABI standard protocol. The PCR products were purified with exonuclease I and shrimp alkaline phosphatase (0.5 U/μl of PCR product) or with QIAquick PCR Purification Kit (Qiagen). Sequences were analyzed in an ABI 377 automated DNA sequencer.

The characterization of a mutation as pathogenic or polymorphic was based on the analysis of the change within a family and/or within 100 control chromosomes. The nomenclature of the mutations follows that outlined by Antonarakis et al. (1998), and we have used the cDNA numbering of the isoform NC11-303 (GenBank accession number AF018082) starting from the first ATG (gene sequence can be found at position chr21: 43334766-43443318 of the draft human genome; June 2002 freeze [Human Genome Browser Gateway]).

Haplotype Analysis

We determined the haplotype of the 21q region, to characterize the origin of a recurrent mutation, as well as to verify whether affected sibs share the same 21q at-risk region. The following markers were used: three intragenic polymorphic markers (dbSNP IDs rs2236451, rs2236474, and rs7499) and *COL18A1*/3′ UTR, as well as microsatellites D21S1897, D21S171, 61.300, 139.000, and 179.500 (proximal to *COL18A1*) and D21S1446 (distal to *COL18A1*).

The microsatellites were analyzed by PCR, which was performed in a total volume of 10 μl (containing 40–60 ng of genomic DNA; 2.5 pmol of each primer; 200 μM dATP, dTTP, and dGTP; 2.5 μM dCTP; 7.5×10^{-4} μCi [α-^{32}P] dCTP; 10 mM Tris-HCl [pH 9.0]; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 0.1% Triton; and 0.1 U *Taq* DNA polymerase). The thermocycling conditions used for amplification consisted of 28 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

The *COL18A1*/3′-UTR polymorphism was analyzed by PCR and SSCP analysis, according to instructions published elsewhere (Sertié et al. 1999).

RT-PCR Analysis of the NC11-728 Isoform

Total RNA was purified from adult lymphocytes, retina, and cultured skin fibroblasts by the guanidium isothiocyanate method (Chomczynski and Sacchi 1987). Fetal-brain RNA was purchased from Research Genetics, and adult-liver and adult-kidney total RNA was purchased from Clontech. RT-PCR was performed with total RNA (1 μg) from each tissue by using the OneStep Reverse Transcriptase systems (Life Technologies). The following pair of primers was used: 5′-CCAGGAGGATGGGTACTGTG-3′ and 5′-AGTCACGGAAGAAGAGGCTG-3′.

The design of these primers was based on the exon structure of the mouse NC11-764 (GenBank accession number AH006757), and they specifically amplify this isoform. RT-PCR products were sequenced in both directions by using the same primers.

Endostatin Plasma Levels

Endostatin plasma concentrations were measured through ELISA. Plasma for endostatin measurements was immediately separated after the drawing of the blood and was frozen at −70°C. We were able to obtain 23 samples, for endostatin plasma measurements, from members of six families—11 patients (3 from family KS1, 1 from KS2, 1 from KS5, 1 from KS7, 3 from KS8, and 2 from KS9) and 12 unaffected individuals (4 from family KS1, 2 from KS5, 2 from KS7, 2 from KS8, and 2 from KS9). Family KS1 includes 12 affected individuals and has been described elsewhere (Sertié et al. 2000). ELISA for plasma endostatin was performed using a commercially available assay (Accucyte; Cytimmune Sciences), according to the manufacturer's instructions. All measurements were performed in duplicate, to ensure the accuracy of the data collected. The kit used has a sensitivity of 1.95 ng/ml and has typical inter- and intra-assay variances of $\leq$10%. The normal values of plasma endostatin in the population that we studied have been estimated as 20.3 ± 11.5 ng/ml (Zorick et al. 2001).

Results

Phenotype and Identification of Pathogenic Mutations

The main clinical features of the patients from the eight unrelated families are summarized in table 1. Note that patient KS7, previously described by Sniderman et al. (2000), does not present a typical phenotype of KS.

In the eight propositi, sequencing analysis of the *COL18A1* gene revealed 20 changes that were considered to be polymorphic because they occurred with a frequency >1% in control chromosomes. All of these polymorphic changes have been described elsewhere (Iughetti et al. 2001). In five unrelated propositi (table

Table 1

Main Clinical Features of the Patients with KS in the Present Study

Patient (Sex)	Age		Myopia	Vitreoretinal Degeneration	Retinal Detachment	Macular Degeneration	Current Status	Other Alterations	Encephalocele
	At Diagnosis of KS	Currently							
KS2-1 (F)	33 years	38 years	Yes	...	Yes (<18 years)	Unknown	Blind	No	Yes
KS2-2 (M)	32 years	37 years	Yes	...	Yes (OD 6 years)	Unknown	Not blind	No	Yes
KS3-1 (F)	2 years	5 years	Yes	Yes	Unknown	Unknown	Not blind	No	Bone defect (visualized through computed-tomography scan)
KS4-1 (F)	2 years 5 mo	21 years	Yes	Yes	Yes (OS 5 years, OD 8 years)	Yes	Blind (at age 5 years)	No	Yes (removed)
KS4-2 (M)	3 d	13 years	Yes (at birth)	Yes	Yes (OS 4 years 6 mo, OD 6 years)	No	20/200 best corrected (both eyes)	No	Yes (removed)
KS5-1 (M)	1 year	6 years	No	No	Yes	No	Blind (at age 1 year)	Epilepsy (onset at 4 years 9 mo)	Yes
KS6-1 (M)	17 years	27 years	Yes	Yes	Yes (OD <2 years)	Yes	Unknown	Yes	Yes
KS6-2 (F)	14 years	24 years	Yes	Yes	Yes	Yes	Unknown	Yes	Yes
KS7-1 (M)	16 d	6 years	Yes	Yes	No	No	Unknown	No	No
KS8-1 (M)	10 mo	10 mo	Unknown	Unknown	Unknown	Unknown	Not blind	No	Yes (removed)
KS8-2 (M)	2 years 6 mo	2 years 6 mo	Yes	Unknown	Unknown	Unknown	Not blind	No	Yes (removed)
KS8-3 (F)	4 years	4 years	Yes	Unknown	Unknown	Unknown	Not blind	No	Yes (removed)
KS9-1 (M)	10 years	12 years	Yes	...	Yes (OS 1 years 9 mo, OD 2 years)	...	Blind (at age 2 years)	No	No
KS9-2 (M)	6 years	8 years	Yes	...	No	Yes	Not blind	No	Yes

NOTE.—OS = *oculus sinister* (left eye); OD = *oculus dexter* (right eye).

Table 2

Pathogenic Mutations

Patient[a]	Mutation(s) (cDNA change)	Region(s)	Consequence(s)	Affected isoforms
KS1[b]	IVS1-2A→T (homozygous)	Intron 1	Splicing—stop codon at 4	Only short form
KS2	No mutation detected	…	…	…
KS3	c2969-2978delCAGGGCCCCC (maternal), c3514-3515delCT (paternal)	Exon 36, exon 41	Frameshift—stop codon at 38; frameshift—stop codon at 42	All isoforms
KS4	c1238-1239insA (maternal), c3514-3515delCT (paternal)	Exon 10, exon 41	Frameshift—stop codon at 13; frameshift—stop codon at 42	All isoforms
KS5	c3514-3515delCT (maternal), c2105delC (paternal)	Exon 23, exon 41	Frameshift—stop codon at 24; frameshift—stop codon at 42	All isoforms
KS6[c]	No mutation detected	…	…	…
KS7	No mutation detected	…	…	…
KS8	IVS1-2A→T (homozygous)	Intron 1	Splicing—stop codon at 4	Only short form
KS9	c3277C→T (homozygous)	Exon 40	Premature stop codon (Q1093X)	All isoforms

[a] For each family, there is only one propositus.
[b] Previously reported by Sertié et al. (2000).
[c] First exon was not analyzed.

2), we identified the causative mutation in both alleles: three were compound heterozygotes, and two were homozygotes. One change, a deletion of CT in exon 41 (c3514-3515delCT), was observed in three unrelated alleles (in families KS3, KS4, and KS5). The five other mutations were an insertion of 1 bp in exon 10 (c1238-1239insA; KS4), a deletion of 1 bp in exon 23 (c2105delC; KS5), a deletion of 10 bp in exon 36 (c2969-2978delCAGGGCCCCC; KS3), a nonsense mutation in exon 40 (c3277C→T; KS9), and a mutation at the acceptor splice site of intron 1 (IVS1-2A→T; KS8). None of these mutations were detected in a 100-chromosome control sample. We analyzed both parents of the propositi from each family and verified that all of them were heterozygous for one of the mutations present in their affected descendants.

Haplotype Analysis

We compared the haplotypes of the alleles carrying the mutation c3514-3515delCT in families KS3, KS4, and KS5. We observed a different haplotype associated with this common pathogenic allele, with no evidence of a founder effect (fig. 2*A*).

We have also compared the haplotypes from families KS8 and KS1 (Sertié et al. 2000), because they also share a common pathogenic mutation in homozygosis (IVS1-2A→T). In this case, we observed a common haplotype (fig. 2*B*).

Patients without Detectable Pathogenic Mutation

In two patients, we did not detect any pathogenic change after sequencing the entire coding region: one patient (KS7) represents an isolated case (Sniderman et al. 2000), and the other patient (KS2) has an affected sib and unaffected parents. In still another familial case (in family KS6; Czeizel et al. 1992), with two affected sibs, we did not detect any pathogenic change in the mother and in any of the 42 exons of the father; therefore, we included this patient in this subgroup representative of cases without a detectable pathogenic change. Segregation analysis with polymorphic markers flanking or within *COL18A1* showed that each pair of sibs (KS2 and KS6) shares a common haplotype for this 21q22.3 region (fig. 2*C*).

Endostatin Measurements

The plasma endostatin levels in the affected patients and in unaffected individuals are summarized in table 3. The plasma endostatin level of individuals homozygous and heterozygous for the IVS1-2A→T mutation were similar and were within the range observed in the control population. Endostatin plasma levels comparable to the control individuals were also observed for two patients (KS2 and KS7) in whom we did not detect any pathogenic change. Levels in the lower range of the control population, <10 ng/ml (Zorick et al. 2001), were observed in the three patients with null mutations (KS5, KS9-1, and KS9-2), in three carriers of null mutations (the parents of patient KS5 and the mother of patient KS9), and in the unaffected mother of patient KS7.

Expression Studies of Variant COL18A1 *mRNAs*

We detected the expression of the variant that corresponds to the mouse isoform NC11-764 in fetal brain, liver, kidney, retina, and fibroblasts (fig. 3). If we assume that this third isoform is transcribed similarly in mice and humans, then we predict that the N-terminal domain of this human isoform has 728 residues (NC11-728), including 235 residues encoded by the usage of the external donor splice site in exon 3 (fig. 1).

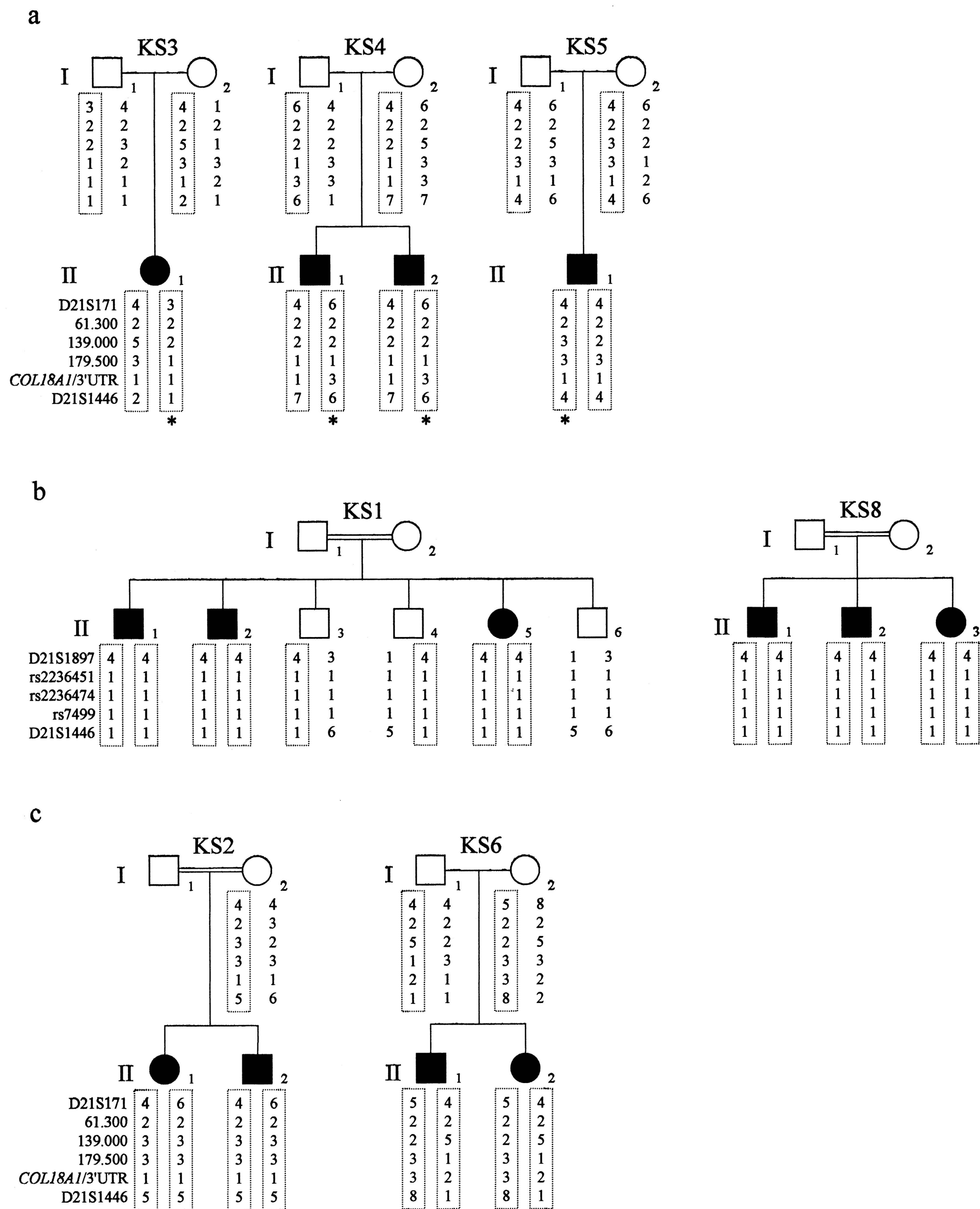

Figure 2 Haplotype for families with KS. *a,* Patients from three unrelated families (KS3, KS4, and KS5), sharing the allele c3514-3515delCT. Asterisks denote the haplotypes harboring the allele c3514-3515delCT. *b,* Patients from two unrelated families, homozygous for IVS1-2A→T. *c,* Familial cases in which no pathogenic change was detected in the patients.

Table 3

Endostatin Levels in Individuals from Families with KS

Family/Patient Number	Clinical Status	Genotype	Endostatin Levels (ng/ml)
KS1-1	Affected	IVS1-2A→T/IVS1-2A→T	19.4
KS1-2	Affected	IVS1-2A→T/IVS1-2A→T	25.5
KS1-3	Normal homozygote	Wild type/wild type	22.5
KS1-4	Mutation carrier	IVS1-2A→T/wild type	22.8
KS1-5	Mutation carrier	IVS1-2A→T/wild type	30.9
KS1-6	Affected	IVS1-2A→T/IVS1-2A→T	18.2
KS1-7	Normal homozygote	Wild type/wild type	21.7
KS2-2	Affected	No mutation detected	17.1
KS5-1	Affected	c3514-3515delCT/c2105delC	8.5
KS5-2	Mutation carrier	c3514-3515delCT/wild type	9.0
KS5-3	Mutation carrier	c2105delC/wild type	9.5
KS7-1	Affected	No mutation detected	26.9
KS7-2	Heterozygous[a]	No mutation detected	8.7
KS7-3	Heterozygous[a]	No mutation detected	17.3
KS8-1	Affected	IVS1-2A→T/IVS1-2A→T	29.3
KS8-2	Affected	IVS1-2A→T/IVS1-2A→T	32.7
KS8-3	Affected	IVS1-2A→T/IVS1-2A→T	21.0
KS8-4	Mutation carrier	IVS1-2A→T/wild type	17.9
KS8-5	Mutation carrier	IVS1-2A→T/wild type	29.3
KS9-1	Affected	c3277C→T/c3277C→T	7.1
KS9-2	Affected	c3277CT/c3277C→T	6.7
KS9-3	Mutation carrier	c3277C→T/wild type	18.4
KS9-4	Mutation carrier	c3277C→T/wild type	9.8

[a] Uncertain status (because the diagnosis in KS7 is under discussion).

Discussion

Identification of Polymorphic and Pathogenic Mutations

The analysis of the coding sequence of *COL18A1* allowed us to identify 20 polymorphic and 6 pathogenic changes. Of the polymorphic alterations, one, D104N (within exon 42, the coding region for endostatin), was found to be associated with predisposition to human prostate cancer (Iughetti et al. 2001); all of the other polymorphic alterations were located in intronic regions or did not change the amino acid residue.

The identification of four frameshift changes, one premature termination, and one splicing mutation in the patients studied confirms that alterations in *COL18A1* cause KS. Except for the splice change IVS1-2A→T, previously reported in another family in our original publication (Sertié et al. 2000), all of the other mutations are novel alterations. The frameshift mutations were located in different regions of the gene (exons 10, 23, 36, 40, and 41) and lead to creation of premature stop codons in exons 13, 24, 38, 40, and 42, respectively. Therefore, all changes probably result in RNA instability (Mendell and Dietz 2001).

The splice change IVS1-2A→T, seen in two Brazilian families (KS1 and KS8), has the same haplotype in the *COL18A1* region, consistent with common ancestry. The deletion c3514-3515delCT, found in three unrelated patients from different populations (two North American and one Brazilian), is embedded in distinct haplotypes at the 21q22.3 region. These findings suggest that this recurrent CT deletion in exon 41 may represent a hotspot site for mutation. We did not identify any sequence motif that could explain a higher mutation rate in this region.

Endostatin Measurements in Patients with KS and Their Relatives

We observed a wide range of variability of endostatin plasma levels in patients with KS and their relatives, in accordance with the distribution previously obtained in the control population. The nonzero endostatin plasma levels (<1 SD of the mean or <10 ng/ml) found in the three affected individuals with null mutations were unexpected, since this molecule was absent in mice with null mutations in collagen XVIII (Fukai et al. 2002). One possible explanation for these discrepancies is the different pathogenic mutational mechanisms involved in each case; alternatively, but less probably, these might be caused by the presence of a cross-reacting molecule, perhaps endostatin derived from the C-terminal region of collagen XV. Endostatin plasma levels should be measured in a larger sample of patients with KS, but the present data do not encourage the use of endostatin plasma levels as a tool for the diagnosis of this syndrome.

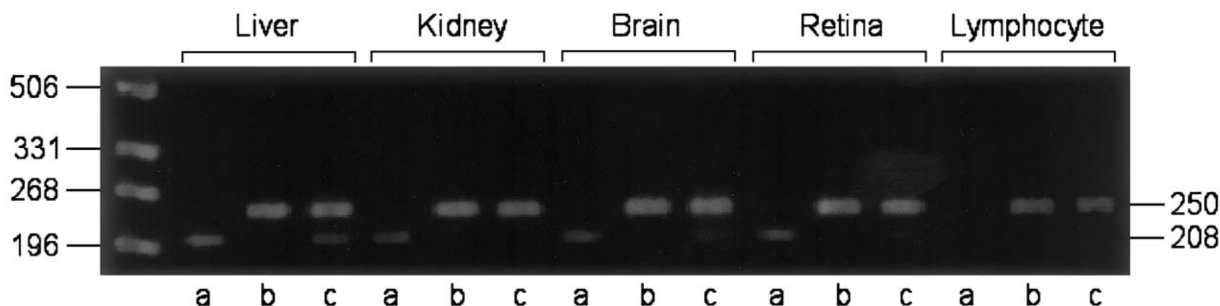

Figure 3 RT-PCR using primers for the human NC11-728 *COL18A1* variant mRNA (208 bp) in different human tissues. *Lane a,* NC11-728. *Lane b,* β-actin (250 bp), used as positive control. *Lane c,* NC11-728 together with β-actin.

Patients without a Detectable Pathogenic Mutation

We were not able to find pathogenic mutations in the coding region in patients from three unrelated families; one (KS7) represented an isolated case, and the two others (KS2 and KS6) represented familial cases. Patient KS7 had some ocular alterations that are comparable to those of KS, but he presented several other clinical features (including a scalp defect in the frontal region, developmental delay, telecanthus, hypertelorism, and high-arched palate) that are not typical of this syndrome. On the basis of the atypical clinical features, we suggest that his phenotype is probably caused by mutations at another locus, and we emphasize that the location of the scalp defect in the occipital region is specific to KS. Menzel et al. (2000) reported discordant 21q22.3 haplotypes in two affected sibs with clinical features of KS, further supporting genetic heterogeneity of this syndrome. In contrast, the patients from the two other families (KS2 and KS6) present both the ocular and occipital defects that are typical of KS; besides, the two affected sibs from each genealogy share a common 21q22.3 region, in agreement with linkage between the *COL18A1* gene and KS. The lack of detectable pathogenic mutation was unexpected, and it is possible that the phenotype in these patients is caused by a different *COL18A1* mutational mechanism that was not detected by the methods used here. Despite the evidence from the linkage studies, we cannot discard the possibility of nonallelic genetic heterogeneity in these patients.

Genotype-Phenotype Correlation

The phenotype of all patients with an identified pathogenic change in *COL18A1* is very characteristic of KS, with occipital scalp defect and severe ocular alterations from birth. Variability in the size of the occipital alteration was observed within and among the different families and does not seem to be associated with the lack of one or all collagen XVIII isoforms. Note that the knockout mice for *Col18a1* do not present this cranial alteration (Fukai et al. 2002).

The premature stop codons in exons 13, 24, 38, 40, and 42 possibly lead to lack of all isoforms of *COL18A1* and endostatin; this lack may be due to mRNA or protein instability (Mendell and Dietz 2001). Interestingly, a frameshift deletion within the C-terminal region of the *Cle1* gene, the homologue of *COL18A1* in *Caenorhabditis elegans,* is associated with a stable product (Ackley et al. 2001).

All seven of the patients from family KS1 who are known to be blind lost their vision after 20 years of age (data not shown). In contrast, we have observed that most patients with mutations that are predicted to cause deficiency of all collagen isoforms are going blind early, in childhood, suggesting that deficiencies of all collagen XVIII forms are associated with a worse ocular prognosis. The human collagen NC11-303 and NC11-493 variants are highly expressed in kidney and liver (Saarela et al. 1998*b*), and we have recently described the presence of the NC11-303 form but not the NC11-493 form in human retina (Sertié et al. 2000). In the present article, we show that the longest human isoform, NC11-728, containing the cysteine-rich frizzled motif, is expressed in the human eye and in other tissues, including liver, as has previously been observed in mice (Muragaki et al. 1995; Rehn and Pihlajaniemi 1995). On the basis of these findings and the apparently more severe clinical course of the eye abnormalities, we suggest that the short isoform (NC11-303) is critical for the maintenance of retinal and eye structure and for the correct closure of the neural tube at the occipital region, but the long isoform (NC11-728) should also play a critical role in the organization and maintenance of the human eye.

We initially hypothesized that the ocular alterations in KS could be related to a deficiency of retinal vascularization and a structural defect in the vitreous humor and retina (Sertié et al. 2000). Indeed, ~80% of mice lacking collagen XVIII and endostatin show abnormal

retinal vessels, probably due to a delayed regression of blood vessels in the vitreous humor, along the surface of the retina after birth; in addition, a reduced number of vitreous collagen fibers were observed along the inner limiting membrane in *Col18a1*$^{-/-}$ mice eyes (Fukai et al. 2002).

None of the patients with mutations in the *COL18A1* gene have any major kidney or liver defects, which is surprising because the three isoforms are highly expressed in these organs (Saarela et al. 1998*a,* 1998*b;* present study). Collagens XVIII and XV belong to the collagen subfamily of multiplexins, with the highest degree of homology in the C-terminal endostatin domain. Collagen XV is immunolocalized in an overlapping distribution with collagen XVIII (Tomono et al. 2002), and mice lacking collagen XV also do not exhibit abnormalities in kidney and liver (instead, they show a mild myopathy) (Eklund et al. 2001). Therefore, it is unlikely that collagen XV plays a compensatory role in the absence of collagen XVIII.

One of the patients (KS4) presented with recurrent epilepsy (detected through electroencephalogram) and thus was the second patient with KS to have this occurrence (Kliemann et al., in press), which had a resultant frequency of 2 in 23 patients with seizures (21 patients from 6 families with a known pathogenic mutation and 2 patients from the family reported by Kliemann et al. [in press]). Deficiency of the NC11 domain of the *cle-1* gene in *C. elegans* is associated with altered axonal migration, and neuronal migration disorders have recently been observed in two unrelated patients with KS (Kliemann et al., in press). These preliminary results suggest that deficiency of collagen XVIII isoforms may predispose individuals to epilepsy, possibly because of abnormal neuronal migration.

In summary, the analysis of the 43 exons of the *COL18A1* gene confirms that mutations in this gene lead to occipital encephalocele and severe ocular alterations and suggests that both the NC11-303 and NC11-728 isoforms of collagen XVIII play critical roles in the maintenance and organization of the human eye. The characterization of other patients with KS will be important to expand the preliminary genotype-phenotype correlation suggested in this report, as well as to confirm the existence of a possible hotspot region in this large molecule.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/ (for polymorphisms in *COL18A1*)

GenBank, http://www.ncbi.nih.gov/Genbank/ (for *COL18A1* mRNA [accession number AF018082] and mouse *Col18a1* gene [accession number AH006757])

Human Genome Browser Gateway, http://genome.ucsc.edu/cgi-bin/hgGateway (for *COL18A1* gene position [chr21: 43334766-43443318; June 2002 freeze])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for KS [MIM 267750])

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