Oscar Takeo Suzuki

Estudo funcional do colágeno tipo XVIII

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Índice

Resumo

A síndrome de Knobloch (SK) é uma doença autossômica recessiva rara, caracterizada por problemas oculares e presença de encefalocele occipital, porém o quadro clínico é variável. Os pacientes apresentam principalmente miopia de grau elevado, degeneração vítreo-retiniana e descolamento de retina; o grau de comprometimento da alteração no occipital também é variável. Nossos estudos mostraram que a SK é causada por mutações no gene *COL18A1*, que codifica o colágeno tipo XVIII. Esse colágeno, uma proteoglicana da matriz extracelular, tem sido estudado principalmente por liberar a endostatina, um fragmento de 20 kDa clivado proteoliticamente de sua porção C-terminal e que possui atividade inibidora da angiogênese. O colágeno XVIII possui três isoformas conhecidas, as quais diferem entre si apenas na porção N-terminal e apresentam padrões de expressão distintos nos tecidos, mesmo estando ubiquamente presentes nas membranas basais epiteliais e endoteliais. Além da endostatina, o colágeno XVIII apresenta outros motivos com funções ainda desconhecidas: um domínio trombospondina, presente em todas as isoformas e um domínio frizzled, encontrado apenas na forma mais longa da proteína. O espectro de variação clinica na SK ainda é incerto, assim como os mecanismos moleculares que levam ao fenótipo. Nosso trabalho teve como objetivos principais a identificação de mutações no *COL18A1* em um número maior de famílias com a SK, estabelecimento de novos protocolos que possam auxiliar no diagnóstico clínico e a avaliação do efeito funcional de variações encontradas na endostatina, domínio de maior conservação do colágeno XVIII. Propusemo-nos ainda a identificar proteínas que interagem com o domínio trombospondina. Apresentamos aqui a caracterização de sete novas mutações no colágeno XVIII em pacientes com SK, permitindo assim uma melhor determinação

do espectro de variação fenotípica da SK. Com base na identificação dessas mutações pudemos incluir problemas neurológicos nos possíveis sinais clínicos presentes na SK ao apresentar pela primeira vez alterações de migração neuronal em pacientes com a síndrome. A ausência de mutações detectadas em três famílias sugere ainda a existência de heterogeneidade genética na SK. Também propomos neste trabalho a utilização da imunohistoquímica em biópsias de pele como teste diagnóstico para essa doença. Nossos resultados mostram também que a variação A48T da endostatina leva a alterações em sua interação com proteínas da matriz extracelular, enquanto a variação polimórfica D104N, previamente associada ao desenvolvimento de câncer de próstata, não leva a um efeito sobre a interação com as proteínas testadas. E, por último, o método de duplo híbrido não foi eficaz para a identificação de proteínas que possam interagir com o domínio trombospondina do colágeno XVIII.

Abstract

Knobloch syndrome (KS) is an autosomal recessive disorder characterized by ophthalmological defects and presence of an occipital encephalocele. Clinical variability is present, however, all patients present high grade myopia, vitreoretinal degeneration and in most cases, retinal detachment; the occipital defect is also variable. Studies show that the KS is caused by mutations in COL18A1, the gene that codes for type XVIII collagen. This collagen is an extracellular matrix proteoglycan and has been the focus of a great number of studies due to its C-terminal domain, endostatin. Endostatin is a 20 kDa fragment that is proteolytically cleaved and possesses a high antiangiogenic activity. Type XVIII collagen is known to be expressed in three isoforms, different among themselves in the N-terminal region. These isoforms have distinct expression patterns, but are present in most basement membranes. Besides endostatin, type XVIII collagen also presents other domains with unknown functions: a thrombospondin domain, found in all isoforms; a frizzled domain, present in the longest isoform. The clinical variability spectrum in KS and the molecular mechanisms that lead to the phenotype are still uncertain. The aim of this study was to identify novel mutations in COL18A1 in additional KS families, to develop biochemical diagnostic tests that could allow the screening of a larger number of patients and to evaluate the effect of naturally found variants in the function of endostatin. We also performed a two-hybrid screening in order to identify proteins that can interact with the thrombospondin domain. The characterization of seven novel mutations in KS patients allowed us to better determine the clinical variability of KS. This work shows for the first time the presence of neuronal migration defects in some KS patients. The lack of detected pathogenic mutations in three families led us to propose the genetic heterogeneity of this syndrome.

We demonstrate the possibility to use immunohistochemistry in skin biopsies as a diagnosis method. Our results also show the altered properties of T48 endostatin in its interaction with some extracellular matrix proteins. The N104 variant, that has been previously associated with prostate cancer, do not present any change in its interaction to the tested molecules. Finally, the two-hybrid system was not a good method to detect interacting proteins with the thrombospodin domain of collagen XVIII.

Introdução

A síndrome de Knobloch (SK; MIM# 267750) é uma condição genética de herança autossômica recessiva aparentemente rara, com 29 casos pertencentes a sete famílias descritos na literatura (Knobloch *et al*., 1971; Czeizel *et al*., 1992; Seaver *et al*., 1993; Passos-Bueno *et al*., 1994; Wilson *et al*., 1998; Sniderman *et al*., 2000; Kliemann *et al*., 2002; Duh *et al*., 2004). Os sinais clínicos característicos da síndrome são: miopia de grau elevado, degeneração vítreo-retiniana, descolamento de retina, catarata, encefalocele occipital (presente na maior parte dos pacientes) e alterações na mácula. Um comprometimento ocular mais grave em alguns pacientes mais velhos mostra que a SK é progressiva.

Outras alterações menos freqüentes também foram encontradas entre alguns pacientes afetados pela SK, tais como: espinha bífida oculta (L5-S3), hipoplasias do pulmão direito, das unhas, maxilar e facial, duplicação do sistema coletor renal com ureter bífido, ponte nasal baixa, estenose pilórica, hiperextensibilidade generalizada das juntas, depressão no couro cabeludo na região frontal do crânio, epilepsia, alterações na morfogênese neuroectodérmica e persistência de vasculatura fetal no desenvolvimento do olho (Czeizel *et al*., 1992; Seaver *et al*., 1993; Passos-Bueno *et al*., 1994; Wilson *et al*., 1998; Sniderman *et al*., 2000; Sertié *et al*., 2000; Kliemann *et al*., 2002; Duh *et al*., 2004).

O gene causador da SK foi mapeado em uma região de 4,3 cM em 21q22.3, através do estudo de ligação em uma família brasileira altamente endocruzada (Sertié *et al*., 1996). Estudos com marcadores adicionais permitiram restringir essa região em cerca de 245 kb, um intervalo contendo 24 ESTs (expressed sequence tags), o gene *KIAA0958* (*POFUT2*) e a extremidade 5' do gene *COL18A1*. O gene causador da

síndrome foi identificado nessa mesma família como sendo o *COL18A1*, onde foi encontrada a mutação c.12-2A>T (numeração de acordo com o RNAm AF018082; http://www.ncbi.nlm.nih.gov/entrez) em homozigoze em todos os afetados pela SK (Sertié *et al*., 2000). O gene *COL18A1* codifica a cadeia α1 do colágeno do tipo XVIII, um colágeno não fibrilar membro da subclasse das multiplexinas (assim como o colágeno do tipo XV) (Rehn *et al*., 1994). Esses resultados mostram que o colágeno XVIII exerce um papel importante na formação e manutenção da estrutura do olho humano.

Mutações em outros tipos de colágeno já haviam sido associadas a alterações oftalmológicas semelhantes àquelas encontradas na SK. Alguns pacientes com síndrome de Stickler e síndrome de Wagner apresentam mutações no gene *COL2A1*. Mutações no gene *COL11A1* também são responsáveis por alguns casos de síndrome de Stickler e síndrome de Marshall (Martin *et al*., 1999; Richards *et al*., 2002).

a) O gene *COL18A1* e seu padrão de expressão

O gene *COL18A1* possui 43 exons que se estendem ao longo de uma região genômica de 102 kb (Rehn *et al*., 1996). Sabe-se que é transcrito e traduzido em pelo menos três isoformas, diferentes entre si na região N-terminal, por meio da utilização de duas regiões promotoras distintas e processamento alternativo do RNA. A forma menor do RNAm é constituída pelos exons 1, 2 e 4 a 43 e codifica um polipeptídeo de 1336 resíduos de aminoácidos, já a forma intermediária apresenta parte do exon 3 e os exons 4 a 43 codificando um polipeptídeo de 1516 resíduos (Saarela *et al*., 1998a). Mais recentemente, constatou-se a existência de uma isoforma maior, com 1751 resíduos de aminoácido sendo codificados pelos exons 3 a 43 (Elamaa *et al*., 2003). Assim sendo, as isoformas do colágeno tipo XVIII diferem no primeiro domínio não-colágeno (NC11),

possuindo 303 (NC11-303), 493 (NC11-493) e 728 (NC11-728) resíduos de aminoácido (figura 1). Uma região de 301 resíduos do domínio NC11 é compartilhada entre as três isoformas, além dos outros domínios da proteína (Saarela *et al*., 1998a).

Estudos de expressão mostram que as variantes do colágeno XVIII são expressas de forma diferente nos tecidos humanos. A isoforma NC11-303 é expressa nos rins, retina, cérebro e na membrana basal de diversas partes do corpo, especialmente dos vasos sangüíneos e epitélios, a isoforma NC11-493 é expressa principalmente no fígado, enquanto a isoforma NC11-728 é expressa em fígado, pulmão, pele, músculo esquelético e baço fetais, pele e timo adultos (Saarela *et al*., 1998a; Saarela *et al*., 1998b; Sertié *et al*., 2000; Elamaa *et al*., 2003). A expressão do colágeno XVIII também foi detectada durante a morfogênese epitelial do pulmão e dos rins (Lin *et al*., 2001). No olho de *Gallus gallus*, a expressão do colágeno XVIII foi detectada no corpo ciliar, disco óptico, córnea e cristalino, utilizando-se hibridização *in situ* (Dong *et al*., 2002). Por meio de imunohistoquímica, a proteína foi localizada na lâmina basal da retina, epitélio pigmentar e cápsula cristalina (Halfter *et al*., 1998).

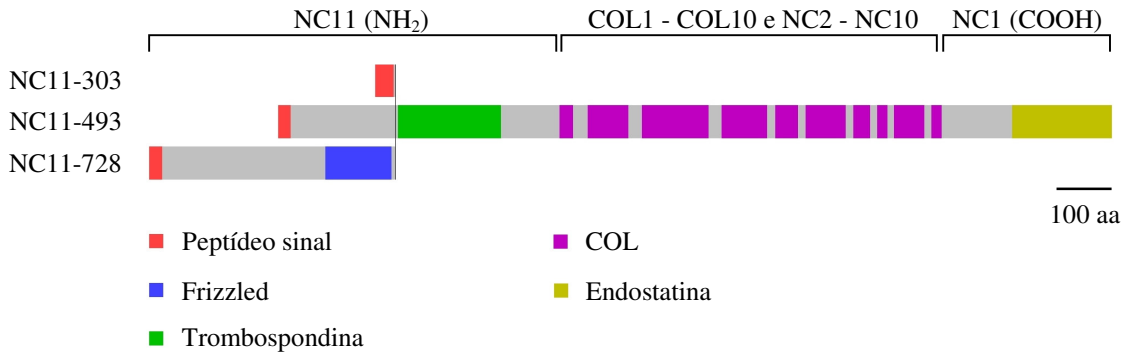

Figura 1. Esquema das isoformas do colágeno XVIII e distribuição dos diferentes domínios conhecidos.

b) A proteína colágeno XVIII

O colágeno XVIII é um componente das membranas basais epiteliais e endoteliais, a proteína forma homotrímeros e possui cadeias laterais de heparan sulfato (Halfter *et al*., 1998; Dong *et al*., 2003), apresentando 11 domínios não-colágeno (NC1 a NC11), interrompidos por 10 domínios colágeno (COL1 a COL10), numerados a partir da extremidade C-terminal (sentido 3'-5'). A presença de múltiplos domínios hélice-tripla Gly-Xaa-Yaa (domínios colágeno) interrompidos por domínios não-colágeno caracterizam as multiplexinas (*multiple triple-helix domains and interruptions*) (Oh *et al*., 1994; Rehn *et al*., 1994). Como discutido abaixo, a partir da análise da seqüência de cDNA e de aminoácidos, vários motivos com possível função biológica foram encontrados na proteína.

No colágeno XVIII de *Gallus gallus*, foi confirmada a ligação de cadeias laterais de heparan sulfato em 3 sítios de ligação para glicosaminoglicanas (GAGs). As cadeias de heparan sulfato mostraram-se necessárias para a ligação do colágeno tipo XVIII às membranas basais. As seqüências de aminoácidos dos sítios de ligação de GAGs estão conservadas em humanos, camundongos e Xenopus, localizando-se nos domínios NC8, NC9 e NC11 (Dong *et al*., 2002; Dong *et al*., 2003).

Na região do domínio NC11 que é compartilhada entre as três isoformas, foi encontrado um segmento (aproximadamente 200 resíduos de aminoácido codificados pelo exon 4) homólogo à trombospondina (Rehn *et al*., 1994; Saarela *et al*., 1998a). A trombospondina é uma glicoproteína secretada com afinidade por diversas moléculas e que apresenta atividade antiangiogênica (Bornstein, 2001). No entanto, não se sabe ainda qual o significado funcional dessa seqüência homóloga à trombospondina no colágeno XVIII, bem como nos colágenos V, IX, XI, XII e XIV, nos quais também é observada (Rehn *et al*., 1994).

A isoforma mais longa do colágeno XVIII possui um domínio rico em cisteínas, homólogo às proteínas frizzled, com aproximadamente 120 resíduos de aminoácido (codificados pela região 3' do exon 3). Os domínios frizzled são receptores para Wnt e estão envolvidos em sua sinalização (Wodarz *et al*., 1998).

O domínio NC1 do colágeno XVIII, com cerca de 300 resíduos de aminoácido, contém em sua região N-terminal um domínio aparentemente responsável pela formação de homotrímeros da proteína (codificada pelos exons 38 e 39), um domínio central com diversos sítios de proteólise (codificado por parte do exon 40) que quando clivados liberam a endostatina, compreendida pela região C-terminal de 184 aminoácidos (codificada por parte do exon 40 e exons 41 a 43) (Sasaki *et al*., 1998).

A endostatina é um fragmento proteolítico de aproximadamente 20 kDa que possui uma forte atividade inibidora de angiogênese e da proliferação de células endoteliais, com capacidade de inibir o crescimento de tumores (O'Reilly *et al*., 1997), por esse motivo a endostatina vem sendo amplamente estudada. A partir da extremidade C-terminal do colágeno XVIII é produzida uma gama de fragmentos de diferentes tamanhos por meio de sítios proteolíticos distintos, variando entre 20 kDa (endostatina) e 38 kDa (domínio NC1 inteiro) (Sasaki *et al*., 1998). A atividade anti-angiogênica foi inicialmente associada à endostatina mas estudos indicam que fragmentos maiores também podem ter alguma atividade relacionada à angiogênese (Yamaguchi *et al*., 1999). Verificou-se mais recentemente em culturas celulares que o domínio NC1 oligomerizado possui função diferente da endostatina no controle de migração e morfogênese celular dependente da matriz extracelular (Kuo *et al*., 2001). Estudos de perfil de expressão gênica e de fosforilação de proteínas mostram que a endostatina é capaz de inibir vias de sinalização associadas à atividade pro-angiogênica e ativar outras

vias associadas à atividade inibidora da angiogênese, tendo assim um extenso efeito na atividade celular (Abdollahi et al., 2004).

Existem também indícios que a endostatina inibe a sinalização da Wnt, cujas vias têm um papel importante na regulação de proliferação, diferenciação, mobilidade e morfogênese celulares (Hanai *et al*., 2002). A endostatina apresenta uma estrutura globular compacta com alta afinidade à heparina, devido a um segmento rico em argininas na superfície da proteína, 6 desses resíduos foram identificados como sendo críticos para a interação com a heparina (Sasaki *et al*., 1999). Hohenester *et al*. (1998) sugeriram uma inibição de angiogênese através da ligação com proteoglicanas. As glipicanas foram mais tarde identificadas como sendo receptores de baixa afinidade para a endostatina (Karumanchi *et al*., 2001).

No domínio COL3 encontra-se ainda um sítio RGD (Arg-Gli-Asp), sendo possivelmente um sítio de ligação às integrinas, o que conferiria ao colágeno XVIII uma atividade na adesão celular (Saarela *et al*., 1998a).

c) O gene *COL18A1* em outros organismos

O gene *col18a1*, presente no cromossomo 10 em camundongos, foi identificado como sendo ortólogo do *COL18A1* humano. O colágeno XVIII de camundongos é encontrado em 3 isoformas que também diferem no domínio não-colágeno N-terminal da proteína. Assim como no homem, essas isoformas resultam da utilização de duas regiões promotoras distintas e o RNA transcrito pelo promotor situado a 3' sofre processamento (*splicing*) alternativo no exon 3 (Rehn *et al*., 1996). Quando comparadas, as seqüências de aminoácidos humana e de camundongo apresentam uma identidade de 79% e uma similaridade de 95%. O domínio de maior homologia é o da endostatina, com identidade de 85% e similaridade de 99% (Saarela *et al*., 1998a).

Em camundongos, o domínio NC11 da isoforma mais longa (NC11-764) também apresenta uma região (cerca de 110 resíduos de aminoácido) com grande homologia às proteínas frizzled (Rehn *et al*., 1995).

Por meio do *knockout* do gene *col18a1* em camundongos, concluiu-se que o colágeno XVIII nessa espécie possui um papel importante na formação e angiogênese da retina. Foram observadas alterações na regressão e maturação dos vasos sangüíneos do vítreo e separação da membrana interna limitante da matriz do vítreo. Marcações imunológicas mostram que o colágeno XVIII está presente nos pontos de ancoragem de colágenos do vítreo à membrana interna limitante (Fukai *et al*., 2002; Hurskainen *et al*., 2005). Mais tarde, também foi verificada a importância da proteína para a manutenção da estrutura do epitélio pigmentar da retina (Marneros *et al*., 2004) e a presença de hidrocefalia em parte dos animais com ausência de colágeno tipo XVIII (Utriainen *et al*., 2004).

Em *Caenorhabditis elegans* o gene *cle-1* é ortólogo do colágeno XVIII presente nos vertebrados e também é expresso em três isoformas distintas. Os colágenos tipo IV e XVIII são os únicos conservados entre *Caenorhabditis elegans* e os vertebrados (Hutter *et al*., 2000). Nesse organismo foram verificadas as influências dos domínios NC1 e endostatina na migração celular e na orientação de axônios (Ackley *et al*., 2001).

d) Correlação entre genótipo e fenótipo em outras famílias com SK

A mutação descrita por Sertié *et al*. (2000) compromete apenas uma das isoformas conhecidas do colágeno XVIII humano (NC11-303). A triagem de mutações no gene *COL18A1* em outras famílias é fundamental para a confirmação da deficiência do colágeno XVIII como a causa da síndrome e possivelmente para o estabelecimento de uma correlação entre o fenótipo e diferentes tipos de mutação.

e) Polimorfismo funcional na região codificadora da endostatina

Recentemente o polimorfismo D104N, presente na endostatina (posição D1437 do colágeno XVIII), foi associado a uma maior predisposição ao câncer de próstata. Indivíduos heterozigotos possuem um risco de desenvolver câncer de próstata 2,5 vezes elevado quando comparados aos homozigotos D104. Simulações mostram que a endostatina N104 é estável, sugerindo uma alteração na função da proteína (Iughetti *et al*., 2001), sendo necessário, portanto, o estudo da proteína alterada. Em uma das famílias com SK em que foi realizada a triagem de mutações, foi encontrada a alteração A48T (posição A1381 do colágeno XVIII), a qual não foi encontrada em controles normais (Kliemann *et al*., 2002). Também não se sabe se a endostatina T48 é alterada funcionalmente.

Objetivos

Os principais objetivos do presente trabalho são os seguintes:

- Identificação de novas mutações no gene *COL18A1* em pacientes com a SK;
- Avaliação do efeito de polimorfismos presentes no domínio endostatina;
- Identificação de proteínas que interagem com o domínio trombospondina do colágeno tipo XVIII.

Capítulo 2

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

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Abstract

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).

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*. 1998b) (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*. [1994a, 1994b]) 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*. 1994a; Saarela *et al*. 1998a, 1998b). 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*. 1994a, 1994b; 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*. 1998a, 1998b). 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*. 1998a, 1998b). 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).

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. 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-32P] dCTP; 10 mM Tris-HCl [pH 9.0]; 50 mM KCl; 1.5 mM MgCl2; 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 ⩽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 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. 2A).

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. 2B).

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. 2C).

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).

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, and 41) and lead to creation of premature stop codons in exons 13, 24, 38, 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.

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*. 1998b), 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*. 1998a, 1998b; 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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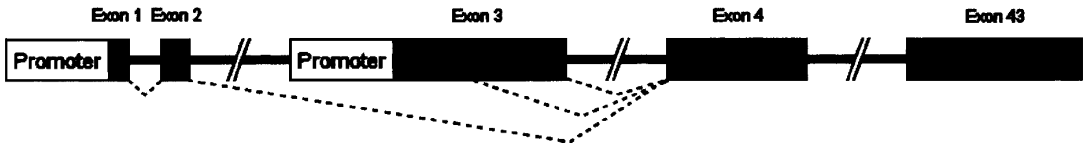

Figure 1 Diagram of the N-terminal region of *COL18A1*, showing the two distinct promoters and the alternate transcription start sites

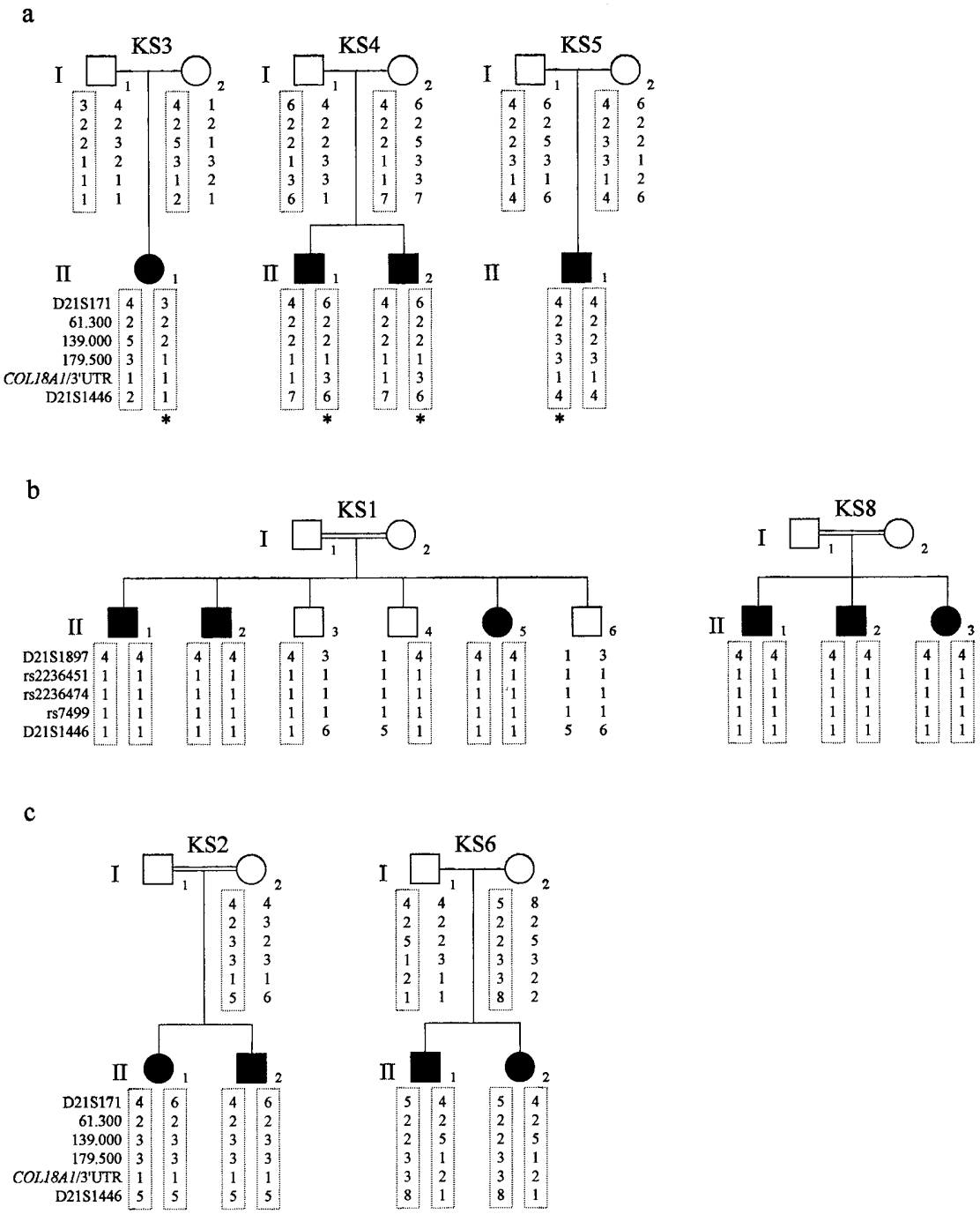

Figure 2 Haplotype for families with KS. *a,* Patients from three unrelated families (KS3, KS4, and KS5), sharing the allele c3514-3515delCT. Asterisks denote the haplotypes harboring the allele c3514-3515delCT. *b,* Patients from two unrelated families, homozygous for IVS1-2A→T. *c,* Familial cases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

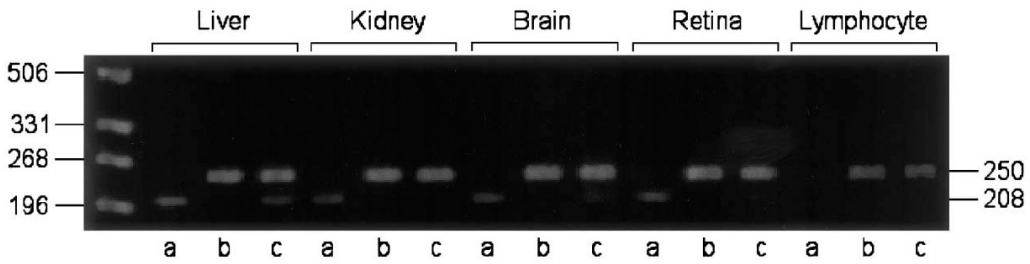

Figure 3 RT-PCR using primers for the human NC11-728 *COL18A1* variant mRNA (208 bp) in different human tissues. *Lane a*, NC11-728. *Lane b*, β-actin (250 bp), used as positive control. *Lane c*, NC11-728 together with β-actin.

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.

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).

Table A
Primer Pairs Used in Amplification of *COL18A1* (5′→3′)

Amplicon	Sense Primer	Antisense Primer
Exon 1	tccgcataaacctgggcttc	gtcagaccccgcaggacc
Exon 2	gtctgacccgtgcctgtc	agaatgaaagggcgggact
Exon 3	tggtgccttctttccttctg	cttccagcagctctgagggt
Exon 3	cggaatggttccacagagc	agtccttggctgtctgggt
Exon 3	tggcccagccgtggcattccta	caagcaccgctgtccctggttaa
Exon 3	gctcccagccagcagctccaac	gaccagcagcaggcagaagaacc
Exon 3	cccaaccacctccaccacgaga	acatcggctgcggttcatccag
Exon 4	cctgcacagccacctcac	atcccagctgctccagtc
Exon 5	actgccacctcgaggacc	ccagtgccccaagtggta
Exon 6	gcgagagcagcgtccttt	cgtgtgcacatgtgtcaca
Exon 7	aaaccaagcaagtctccacc	caccatcacacaaacacacg
Exon 8	aggactgaaagcgtttggg	acagaggggcttcatcagg
Exon 9	agcctgggactctggagg	cagcctctccccctcctac
Exon 10	tggggtgcatttccatgtag	acagtctcctcactgggtcg
Exon 11	gcactgagagtgctgggtg	atcataccctgggcacgc
Exon 12	cttcctgtgttggcccttg	tggagctgatacttctgggc
Exon 13	ctcgatatgcagcttgcg	cagctgaatcttgggggtc
Exon 14	gttctgggggtggtggtc	gggtgttcacccctcactc
Exon 15	gcccagataacctgttaaccc	tcctggcatttccccttc
Exon 16	atgtgccttcctctgtccac	ccaccctctttgggggtc
Exon 17	gaaaacgtgtctacgctggg	tctgtgagtgagatgccagg
Exon 18	catgtcccaccctcctctc	tctcagggacactctcctgc
Exon 19	tactagcgggcttttcttgc	caagatgtcgagacagggc
Exon 20	ctgggtaactcacccttccc	agctgatctccgcaggtg
Exon 21	gactcctcgtgggggtcc	cacatctcgtgccagctttc
Exon 22	atcttcatcagagccatccc	agctgaggccaaaccttctg
Exon 23	aaggccacagtccacagc	agggcctgtcctgacctg
Exons 24/25	aagtcgctcgagtccagttg	ctgctctgtgctaccaggtg
Exon 26	ctgtcgggggagatggag	ccaacagtggtcatctggg
Exon 27	ctctgggtgaggctttgtg	tccccaggctgtctccac
Exon 28	ctcagagaggctgccagg	acagcacaagctcagcagc
Exon 29	cagggtctgccccactaag	cctctgggcatgtcaccc
Exon 30	gtgtgaggctgagcgtgg	taagtggcctctgggaggag
Exon 31	gaactgtctctgctcccagc	ctgtggggctctgtgtgac
Exon 32	gacccctgagtggtggtg	aaggaaggtggggctctg
Exon 33	agtcctccctggcacattc	gtggcggctctaggtgtg
Exon 34	tagaagggcctcaggcaaac	agaaattgggagcagccac
Exon 35	cgagccctattctatgcagc	gtcagtgcacccacaccc
Exon 36	accggtgactcagaggctg	ctgcaggagagaaaccaagg
Exon 37	gtaagtcagtggggagtggg	tgggagagtgtctctgaggg
Exon 38	ctgaaacgggcattccttc	ctctcgccttccttaagcc
Exon 39	cgtaccctggcacaggcttggag	ggagggcaatgagcggcagacac
Exon 40	tcacccagcccagaggag	cttctgctgccgagagcc
Exon 41	gcctgtccacacaggtgc	taggcgccagtgtctgtaac
Exon 42	acaaacacccacacccatc	gaaactgcagataggagccc
Exon 43	caggttgtgggagcctctg	agtatggcagccaggtcc
61.300	caggaacgaccaaaacttcc	aaggagaacccacgacctg
139.000	gcaggcagtacagagttccc	aacatcctccccacacacc
179.500	cacgtgtgttgcatatgtgtg	catctggcttctatggcagg

Capítulo 3

Evidence of neuronal migration disorders in Knobloch syndrome: Clinical and molecular analysis of two novel families

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Abstract

Knobloch syndrome is an autosomal recessive disease characterized by the early onset of severe myopia, vitreoretinal degeneration with retinal detachment, macular abnormalities, and midline encephalocele, mainly in the occipital region. Intra and interfamilial variability is present since the encephalocele is not found in all patients, and the degree of myopia is variable. Analysis of the associated malformations suggests alterations during early neuroectodermal morphogenesis. Only 24 cases have been reported. Recently, the gene responsible for the syndrome, mapped to 21q22.3, was identified. The present study reports on four new cases, revealing the existence of neuronal migratory defects associated with the disorder for the first time.

Introduction

Knobloch syndrome (KS) is an autosomal recessive disease characterized by the early onset of progressive, severe myopia, vitreoretinal degeneration with retinal detachment, macular abnormalities, elevated incidence of congenital cataract, midline encephalocele, mainly in the occipital region, and normal cognitive development (Cohen and Lemire, 1982).

Knobloch and Layer (1971) were the first to describe a family in which five of ten sibs from unrelated parents were affected. Currently, only 24 cases are known (see Sniderman *et al*., 2000, for review). Recently, a mutation in the collagen XVIII gene (*COL18A1* gene), mapped to 21q22.3, has been shown to cause the syndrome (Sertié *et al*., 1996; Sertié *et al*., 2000).

The present study reports on four cases from two unrelated families, emphasizing neuronal migration disorders, not previously associated with the syndrome.

Clinical Reports

First Family

The parents are second-degree cousins; the mother aged 40 years, and the father 36 years old. The mother suffered a miscarriage in the first trimester of pregnancy and has borne three children: two boys (patients 1 and 2) and a girl who is currently 12 years old and is physically, neurologically, and ophthalmologically normal. The parents state that no members of previous generations have worn glasses or exhibit ophthalmological alterations. However, we did not evaluate other family members.

Patient 1

This 11-year-old boy was born via vaginal delivery, at term, after an uneventful pregnancy. Birth weight was 3,200 g. The mother was aged 30 at the time of birth. Bilateral cataract and exotropia were noted at birth. The psychomotor milestones were normal but the patient never exhibited good visual acuity, and vision was lost in the left eye at the age of 1 year and 9 months. At that time, a diagnosis was made of retinal detachment in the left eye with anterior uveitis and bilateral cataract. At the age of 2 years, the patient suffered right retinal detachment. At the age of 3 years and 11 months, the child was submitted to lensectomy and posterior vitrectomy but never recovered his vision, remaining amaurotic with progressive atrophy of both eyeballs (phthisis bulbii).

At admission, the patient's physical and neurological examinations were normal. Weight was 32 kg, height 141.5 cm, and head circumference 54.5 cm without scalp alterations. Cognitive development was normal and currently the patient is a gifted student attending a school for the visually handicapped.

Cranial radiography was normal revealing no alteration of the skull bones. Brain MRI, vertebral column radiography, and an ultrasound scan of the urinary tract gave normal results.

Ophthalmological evaluation revealed enophthalmos in the right eye with band keratopathy and a shallow anterior chamber. In the left eye, band keratopathy, a flat chamber, posterior synechiae, and a totally opaque lens were found. An ultrasound scan showed complete bilateral retinal detachment and phthisis bulbii; fundoscopic examination could not be performed owing to opacity of the humours.

Patient 2

This 7-year-old boy is the younger brother of patient 1. Pregnancy was normal with a vaginal delivery at term. Birth weight was 3,800 g. A large head was reported as there was a midline occipital bone defect, which was confirmed by three-dimensional CT scan reconstruction of the skull (Fig. 1A). At the age of 14 months severe myopia was detected and glasses were prescribed. Serial cranial CT scans performed annually revealed nonhypertensive, supratentorial, ventricular dilatation, a moderate and stable retrocerebellar arachnoid cyst, and a defect in the midline portion of the occipital bone. Psychomotor development was normal.

Height was 115 cm, weight 20 kg, and head circumference 55.5 cm (above the 98th centile). Physical and neurological examinations were normal. Cognitive level was adequate. Currently, the patient is already literate and is a gifted student.

Ophthalmological evaluation revealed normal biomicroscopy. Fundoscopic examination of the right eye showed diffuse changes in the retinal pigment epithelium and macular coloboma with diffuse myopic degeneration. Fundoscopic examination of the left eye also revealed diffuse changes in the retinal pigment epithelium, and macular hypoplasia. Refraction evaluation showed myopia in the right eye (-8.50 sphere) while the left eye exhibited compound myopic astigmatism (-6.50 sphere; -2.00 cylinder at 180 degrees).

Cranial MRI revealed many subependymal, heterotopic nodules in the lateral ventricles, more evident on the left side (Fig. 1B). A urinary tract ultrasound scan and vertebral column radiography were normal.

Second Family

The parents are first-degree cousins; the mother aged 37 and the father 40 years old. Both are healthy and their physical and neurological examinations were unremarkable. The mother's ophthalmological examination revealed compound hypermetropic astigmatism, pterygium of the right eye, and emmetropia. The father's examination showed mild myopia in both eyes (-0.50 sphere). Fundoscopic examinations and biomicroscopy were normal. The mother reported three pregnancies, giving birth to three children: two girls (patients 3 and 4) and a boy who died at the age of 7 months due to an acute infectious process. At the time, his psychomotor development and visual contact were normal.

Patient 3

A 19-year-old girl; born at term by vaginal delivery when her mother was 18 years old. Pregnancy was normal. Birth weight was 3,100 g. Bilateral horizontal nystagmus and early bilateral exotropia, more intense during the visual fixation periods, were noted at birth. A small area of alopecia with a flat, wine-colored hemangioma also was observed at the midline of the occipital region at birth. At the age of 2 years, severe myopia was diagnosed in both eyes (-9.0 Diopters). At the age of 13 years, retinal detachment occurred in the patient's left eye with irreversible loss of vision.

Height was 166 cm, weight 53 kg, and head circumference 53 cm. Physical and neurological examinations were normal. Cognitive level was preserved and the patient is a gifted student. Cranial CT scan, EEG, vertebral column radiography, and a urinary tract ultrasound scan were normal. Cranial MRI showed a basilar impression. The three-dimensional CT scan reconstruction of the skull showed a depression at the midline of the occipital bone without discontinuity.

Ophthalmological evaluation revealed bilateral, horizontal nystagmus and exotropia, together with compound myopic astigmatism in the right eye (-12.25 sphere; -3.75 cylinder at 157 degrees), and amaurosis of the left eye.

Patient 4

This 13-year-old girl is the youngest sister of patient 3. Pregnancy and delivery at term were normal. Birth weight was 4,350 g. At birth, a slight prominence and alopecia were noted at the midline of the occipital region, together with bilateral, horizontal nystagmus and esotropia. A cranial CT scan performed at the age of 12 months showed moderate, nonhypertensive, supratentorial ventricular dilatation. An ophthalmological evaluation diagnosed severe myopia, and the patient has worn glasses since then. Over the last 2 months the patient has suffered several, generalized grand-mal seizures, medicated with Phenobarbital (100 mg/day). Height was 148 cm, weight 51 kg, and head circumference 54 cm. Physical and neurological examinations were normal. The patient is a gifted student and her cognitive level is preserved.

EEG, vertebral column radiography, and a urinary tract ultrasound scan were normal. A three-dimensional reconstruction of the skull by cranial CT scan showed a small discontinuity at the midline of the occipital region (Fig. 2A), moderate, nonhypertensive, supratentorial, ventricular dilatation, and slight calcification of the right parietal lobe. Cranial MRI disclosed moderate ventricular dilatation and a pachygyric area in the right frontal lobe, together with a heterotopic nodule on the subpendymal surface of the right lateral ventricle (Fig. 2B). Ophthalmological evaluation revealed severe myopia (OD: -14.50 sphere; OS: -13.25 sphere), bilateral, horizontal nystagmus and esotropia, iris pigments in the anterior lens capsule of the

right eye, and diffuse changes in the retinal pigment epithelium with macular hypoplasia in both eyes, together with myopic degeneration.

Methods and Results: Molecular Analysis

DNA was isolated from whole peripheral blood samples using standard techniques (Miller *et al*., 1988). Segregation analysis was performed employing four markers (the microsatellite D21S1897 and 3 SNPs - dbSNP IDs: rs2236451, rs2236474, and rs7499, http://www.ncbi.nlm.nih.gov/SNP) located within or near the *COL18A1* gene in the two families. Both parents, the two affected sibs from each family, and the normal sister from the first family were included in the analysis. Mutation screening in the 43 coding exons of *COL18A1* is being performed using PCR-SSCP as described elsewhere (Sertié *et al*., 2000). PCR products displaying a mobility shift were sequenced in an automatic sequencer (ABIPrism377, PE Biosystems Foster City, CA) according to the manufacturer's instructions.

The two affected sibs in each family share a common haplotype. These results corroborate the hypothesis that mutations in the *COL18A1* gene may be responsible for the patients' disease in the two families. A total of 27 and 25 of the 43 exons were screened by SSCP analysis in the first and second families, respectively. Twelve alterations, confirmed through sequence analysis, were found. All were shown to be polymorphisms, except for one; a nucleotide substitution from G to A at position c.4181 (AF018081; gi: 2920534) (Fig. 3). This mutation (A1381T) was present in both alleles of the two affected sibs from the second family and it was not detected in 100 control chromosomes. We are currently performing functional studies to elucidate if this

mutation is responsible or not for the KS phenotype. Presently no pathogenic change has been detected in the first family.

Discussion

Knobloch and Layer (1971) described five sibs showing early, progressive, severe myopia, associated with occipital encephalocele in four. Cognitive development was preserved in all patients. Since then, 19 further patients with similar manifestations have been reported in the literature (see Sniderman *et al*., 2000, for review). The progressive nature of the ocular defects in KS is the most prominent symptom and such patients are usually referred to ophthalmologists. Vitreoretinal degeneration with severe myopia of early onset and eventual retinal detachment are universal in this syndrome, which has a poor prognosis, despite surgical treatment or prophylactic cryotherapy (Knobloch and Layer, 1971; Wilson *et al*., 1998). Ophthalmological evaluation has been performed in 15 of the 24 reported patients, 12 of which presented retinal detachment. The five patients described by Knobloch and Layer (1971) were followed up for 10 years by Cook and Knobloch (1982), and unilateral or bilateral retinal detachment was found in all of them between the age of 7 and 11 years. Retinal detachment between the age of 2 and 5 years has been reported in two patients by Czeizel *et al*. (1992) and in another by Seaver *et al*. (1993). The three remaining patients who did not show retinal detachment reported by Seaver *et al*. (1993), Wilson *et al*. (1998), and Sniderman *et al*. (2000) were only 2, 3, and 4 years old, respectively, at the time of the report. However, irreversible ocular damage can occur in the first month of life as found in the patient reported by Wilson *et al*. (1998). One of our patients (patient 1) presented retinal detachment in the left eye at the age of 1 year and 9 months, and in the right eye at the

age of 2 years, with irreversible amaurosis; the other patient (patient 3) presented retinal detachment in the left eye at the age of 13 years with irreversible, unilateral loss of vision.

Neural tube closure defects are prominent and have been described in 22 of the 24 reported cases. There is considerable controversy concerning their classification as scalp defects or true encephalocele. When present, encephalocele is mild and atretic. Pathological analysis was performed in five patients who underwent corrective surgery. Of these, the two patients reported by Czeizel *et al*. (1992) presented only meningocele. The histological examination of both patients reported by Seaver *et al*. (1993) revealed heterotopic neuronal tissue in the occipital scalp defect, with a descending column of fibrous tissue extending to the periosteum containing heterotopic hair shafts and sweat glands. In the single patient reported by Wilson *et al*. (1998), histologic examination revealed a cyst-like space lined by multilayered, unremarkable meningothelium, containing small amounts of neuroglial tissue in the wall.

Several somatic malformations have been encountered in seven of the twenty-four reported cases. It is unclear whether these features constitute part of the clinical spectrum of the syndrome. Knobloch and Layer (1971) reported a patient with Scimitar syndrome. Czeizel *et al*. (1992) found many somatic malformations like spina bifida occulta of L5-S3, caried teeth, asymmetrical chest, unusual palmar creases, finger nail hypoplasia, and bifid ureter in their two patients. Seaver *et al*. (1993) encountered short palpebral fissures, bilateral epicanthic folds, flat nasal bridge, midface hypoplasia, and generalized hyperextensibility of the joints in their two patients. Wilson *et al*. (1998) reported a patient with a single umbilical artery, patent ductus arteriosus, pyloric stenosis, micrognatism, and lop ears. The patient reported by Sniderman *et al*. (2000) presented hypertelorism, telecanthus, flat nasal bridge, and a high-arched palate. None

of our patients exhibited malformations, nor did the 12 patients reported by Passos-Bueno *et al*. (1994).

Ocular development and neural tube formation take place during the first 3 weeks of embryonic life. Thus, any pathological event occurring during this period may adversely affect the embryogenesis of both structures (Seaver *et al*., 1993).

The neuronal migration disorders found in two of our unrelated patients suggest that this cerebral malformation may constitute part of KS. Despite this cerebral defect, the psychomotor development and neurological examinations of both patients were normal. However, patient 4 presented epilepsia, not previously reported in the syndrome. This patient's imaging examinations showed a small calcification of the right parietal lobe associated with nodular heterotopia and moderate, lateral, ventricular dilatation. Whether the epilepsy in this case is related to the heterotopia or to the calcified lesion, itself possibly consequent to a calcified cysticercus, is a matter of speculation.

Migration disorders occur after neural tube closure during embryogenesis. Sertié *et al*. (2000) showed that KS is caused by mutations in the *COL18A1* gene, and its absence might impair embryonic cell proliferation and/or migration as a primary or secondary effect. These authors also stated that collagen XVIII plays a critical role in the maintenance of retinal structure and in neural tube closure based on the phenotype of this syndrome. In addition to a structural function of the collagen XVIII, its COOH-terminal domain (NC1) is proteolytic cleaved and produces endostatin (ES), a potent antiangiogenic agent (O'Reilly *et al*., 1997). Ackley *et al*. (2001) have recently identified a type XVIII collagen homologue, designated as cle-1, in the nematode *Caenorhabditis elegans*. Deletion of the NC1 domain of the cle-1, containing the ES region, results in cell and axon migration defects. These authors also demonstrated that

monomeric ES might inhibit the promigratory activity of the NC1 domain, indicating that the cle-1 NC1/ES domain regulates cell and axon migration in *C. elegans*. Kuo *et al*. (2001) also showed that the collagen XVIII NC1/ES induces motility of nonendothelial mammalian cells, as those from embryonic kidney. Therefore, the present report further supports a role of collagen XVIII in migration of nonendothelial cells and it is showing for the first time a role of this collagen in neuronal migration in vivo.

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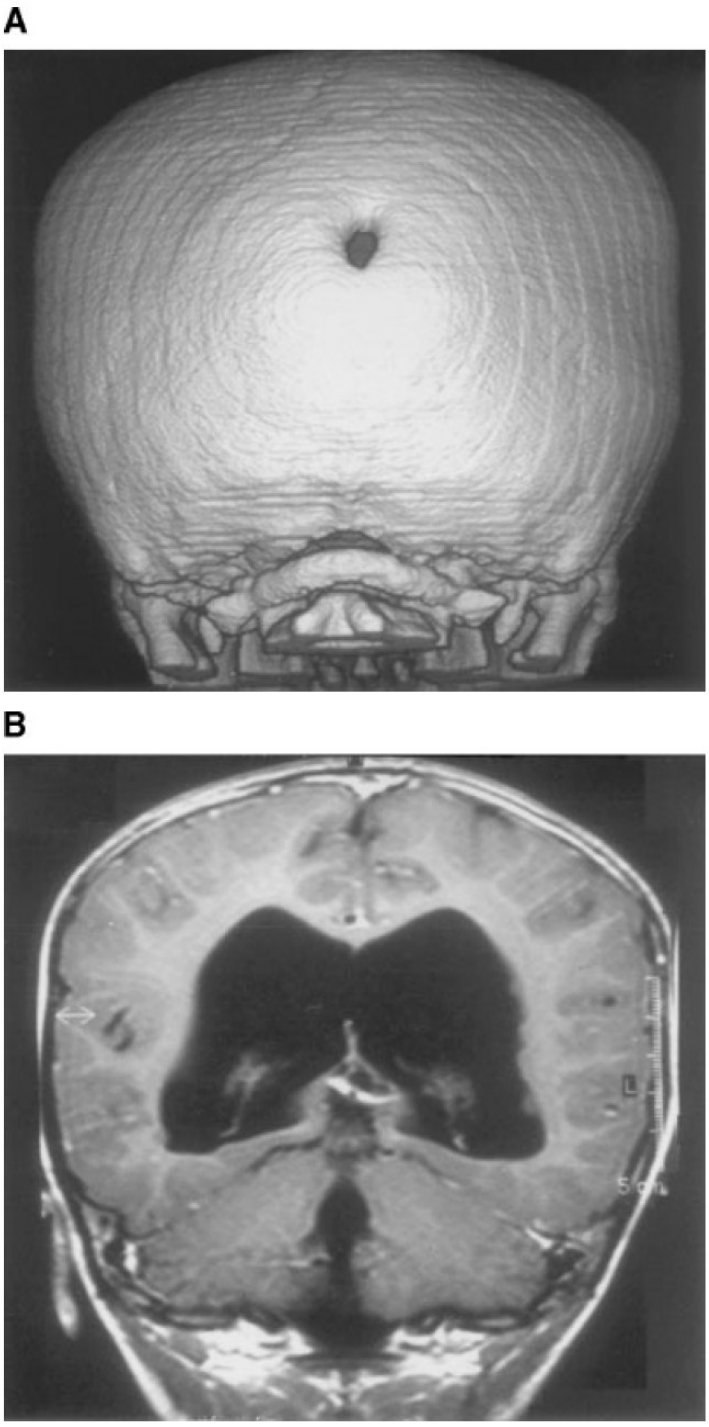

Fig. 1. Patient 2. **A**: A three-dimensional CT scan reconstruction of the skull revealing a discontinuity at the midline of the occipital bone. **B**: T1-weighted MRI (coronal section) showing bilateral, subependymal, heterotopic nodules, more evident on the left side.

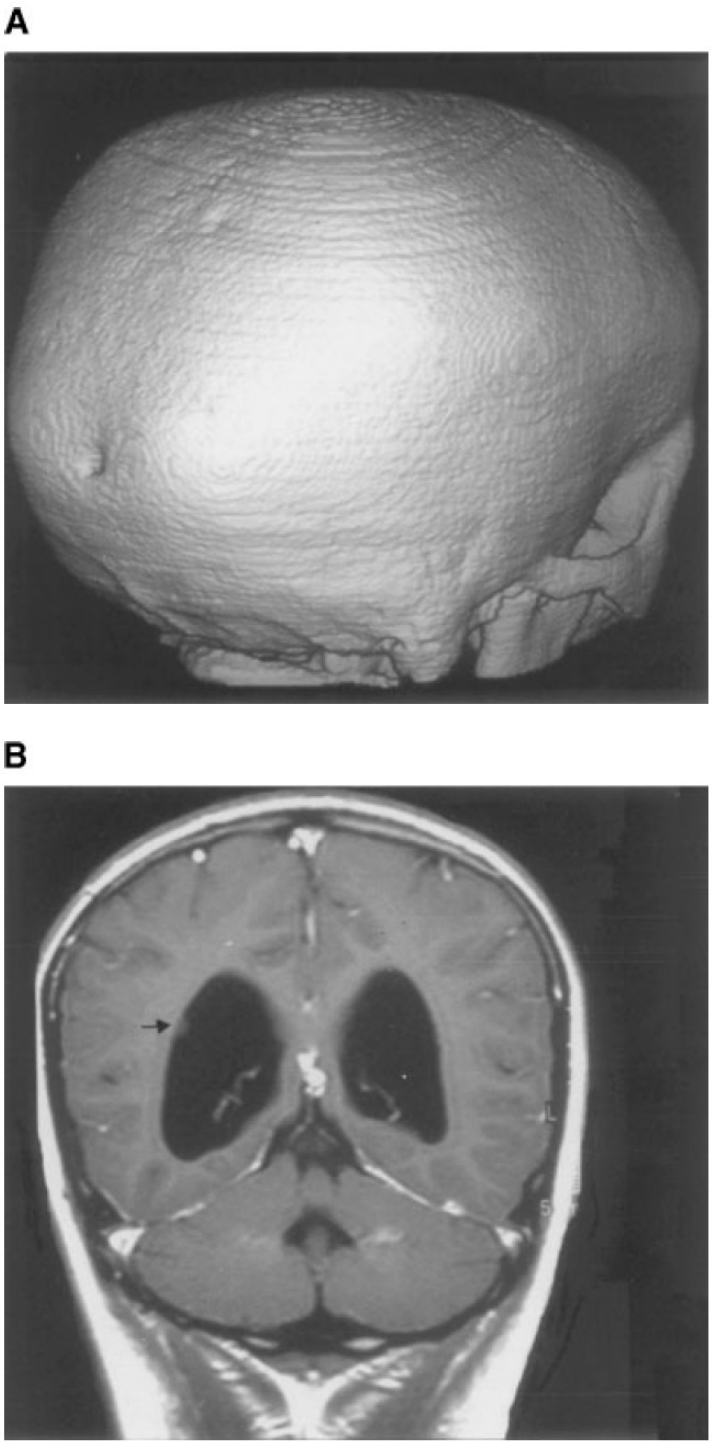

Fig. 2. Patient 4. **A**: A three-dimensional CT scan reconstruction of the skull showing a discontinuity at the midline of the occipital bone. **B**: T1-weighted MRI (coronal section) showing nodular, subependymal heterotopia on the right side (arrow).

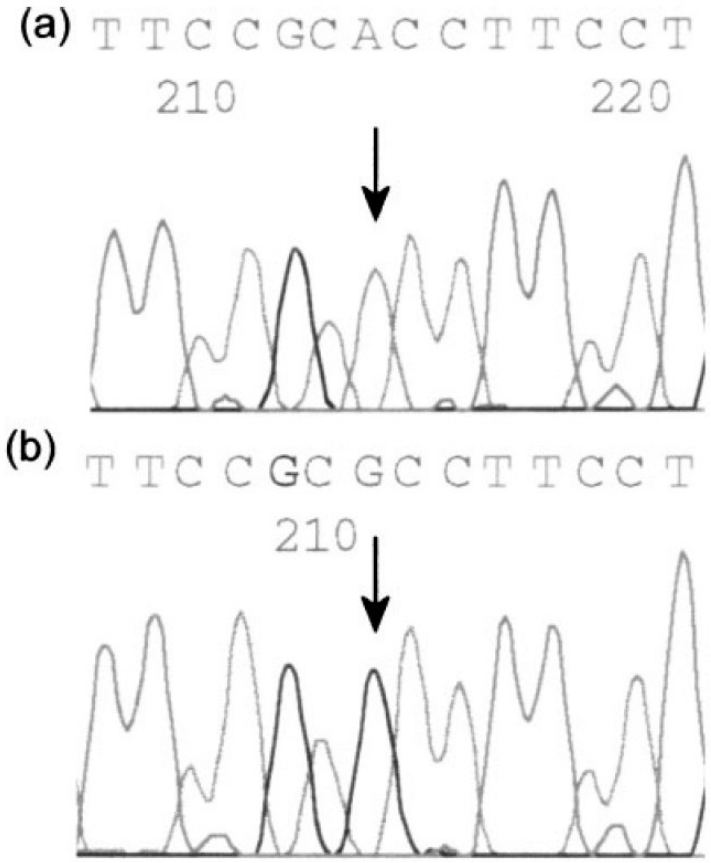

Fig. 3. G to A substitution at position c4181 (gi: 2920534) identified in patients from the second family: **(a)** mutated type; **(b)** wild type.

Capítulo 4

How Pathogenic is the p.D104N/Endostatin Polymorphic Allele of *COL18A1* in Knobloch Syndrome?

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Knobloch syndrome (KNO1; MIM# 267750) is an autosomal recessive disease caused by mutations in the *COL18A1* gene (MIM# 120328), which encodes the type XVIII collagen (Sertié *et al*., 2000; Suzuki *et al*., 2002). The proteolytic cleavage of type XVIII collagen produces the endostatin fragment, a potent angiogenesis inhibitor (O'Reilly *et al*., 1997). This fragment has been the subject of intense research due to its therapeutic potential, but the mechanism of action is still unknown. Iughetti *et al*. (2001) described a polymorphic residue change in endostatin at position 104 (p.D104N; c.4309G>A in exon 42 or D1437N; RefSeq NM_030582; nomenclature of sequence variation according to den Dunnen and Paalman (2003) and Human Genome Variation Society recommendations (www.hgvs.org/mutnomen/checklist.html). This variant affects an aspartic acid residue that is conserved among vertebrates and nematodes, as well as in the collagen XV endostatin-homolog. Based on the crystal structure model of endostatin, we observed that the p.D104 residue is located in the surface of the molecule and its charge is modified by the presence of the allele p.N104. In addition, we also found a suggestion that this change predisposes to prostatic adenocarcinoma. These data lead us to suggest that this polymorphism has consequences in the function of the protein.

Recently in *Human Mutation*, Menzel *et al*. (2004) reported a mutation screening performed in the *COL18A1* and COL15A1 (MIM# 120325) genes of the families described by Czeizel *et al*. (1992) and Wilson *et al*. (1998). The authors detected the insertion c.3363_3364insC present in heterozygosity in a family of Hungarian origin; the only change found on the other copy of the gene was the polymorphism p.D104N of endostatin. The authors also showed that the p.N104 endostatin presents a reduced affinity towards laminin, which reinforces our previous findings that this change is functionally important. Based on these observations, they

suggested that this variant, in combination with a null mutation in the other copy of the gene, causes Knobloch syndrome.

To confirm this proposition, we genotyped the p.D104N polymorphism in seven KNO patients with two null mutations and 12 of their parents (unaffected). Exon 42 of each person was amplified by PCR, the product was digested by MseI and the fragments were separated on a polyacrylamide gel. Genotypes were confirmed by sequencing. None of the patients presented the p.D104N polymorphism and only one parent (the mother of Patient KS4) was heterozygous for this change. This woman also bears the pathogenic mutation c.1238_1239insA in exon 10 of *COL18A1*, which was transmitted to her two affected children with KNO1. Therefore, the allele p.N104 is in trans with the frameshift insertion in exon 10. This woman is healthy, with no clinical signs of the syndrome, and therefore it is very unlikely that the p.D104N mutation in *trans* with another null mutation causes KNO1. The haplotypes constructed using markers flanking the *COL18A1* gene in this woman and her children do not show any recombination in this region (Suzuki *et al*., 2002).

The p.N104 allele has a frequency of 6% in our population and homozygous individuals are perfectly healthy, which supports the hypothesis that this mutation might not be very harmful, despite the possibility of altering the function of endostatin or collagen XVIII. A total of 17 families with KNO patients are under investigation in our laboratory. Six of them are Brazilian, while all the others have been referred by international clinicians (seven Europeans and four North Americans). We found null mutations in both alleles in seven of them, reinforcing the idea that the disease depends on knockout of the two alleles. Considering the frequency of null alleles q_n and the frequency of the polymorphism q_p, according to the hypothesis proposed of Menzel *et al*. (2004), the incidence of KNO1 should be $q_n^2 + 2q_nq_p$. For a disease as rare as KNO1,

this would imply a frequency of null/N104 patients at least 20 times higher than null/null patients, given a polymorphism frequency ranging from 0.02 to 0.06 (Iughetti *et al*., 2001; Liu *et al*., 2003) and a hypothetical null allele frequency of 0.002. On the other hand, the p.N104 allele, for which there is a growing amount of evidence that it is functional, might represent an at-risk or modifier allele for complex disorders in which angiogenesis play an important role, such as cancer.

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Capítulo 5

Mutations in Collagen 18A1 (*COL18A1*) and their relevance to the human phenotype.

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Abstract

Collagen XVIII, a proteoglycan, is a component of basement membranes (BMs). There are three distinct isoforms that differ only by their N-terminal, but with a specific pattern of tissue and developmental expression. Cleavage of its C-terminal produces endostatin, an inhibitor of angiogenesis. In its N-terminal, there is a frizzled motif which seems to be involved in Wnt signalling. Mutations in this gene cause Knobloch syndrome (KS), an autosomal recessive disorder characterized by vitreoretinal and macular degeneration and occipital encephalocele. This review discusses the effect of both rare and polymorphic alleles in the human phenotype, showing that deficiency of one of the collagen XVIII isoforms is sufficient to cause KS and that null alleles causing deficiency of all collagen XVIII isoforms are associated with a more severe ocular defect. This review besides illustrating the functional importance of collagen XVIII in eye development and its structure maintenance throughout life, it also shows its role in other tissues and organs, such as nervous system and kidney.

Introduction

Type XVIII collagen is a non-fibrillar proteoglycan collagen which, together with collagen XV, form the subgroup of multiplexins (multiple triple-helix domains with interruptions) within the collagen family (Rehn and Pihlajaniemi *et al*. 1994, Oh *et al*. 1994, Saarela *et al*. 1998). This protein forms homotrimers and contains an N-terminal non-collagenous domain (NC-11), 10 collagenous repeats alternated with 9 non-collagenous repeats and a C-terminal non-collagenous region, NC-1 (Saarela *et al*. 1998). The N-terminal region presents thrombospondin-1-like and frizzled motifs. This last motif is characterized by a conserved region of 120 amino acids containing a sequence of 10 invariantly spaced cysteines, the cysteine-rich domain or CRD (Xu and Nusse 1998). The frizzled domain binds Wnt signalling molecules, which are involved in cell proliferation, polarity and differentiation in various developmental processes (Wodarz and Nusse 1998). However, the function of the frizzled motif in collagen XVIII molecule is still not known (Lin *et al*. 2001, Elamaa *et al*. 2003). The C-terminal region NC1, which contains a trimerization domain and the last 184 amino acid residues, can release endostatin (20 kDa) and endostatin-like fragments by its cleavage with elastase and/or cathepsin L (Wen *et al*. 1999, Felbor *et al*. 2000, John *et al*. 2005). These fragments can be detected in blood plasma and tissue extracts (Sasaki *et al*. 1998), during physiological or pathological processing of collagen XVIII (Zorick *et al*. 2001, Strik *et al*. 2001, Dhar *et al*. 2002). Endostatin, first identified as a potent inhibitor of endothelial cell proliferation and migration, with the ability to reduce tumor growth, seems to play several other functions, including apoptosis induction (Dhanabal *et al*. 1999, Dixelius *et al*. 2000, Schmidt *et al*. 2004). Endostatin/collagen XVIII also acts in neuronal and renal epithelial cell migration (Ackley *et al*. 2001, Karihaloo *et al*.

2001, Lin *et al*. 2001) and interacts with at least two cell surface receptors (glypican and integrins), but its downstream targets are still unknown (Rehn *et al*. 2001, Karumanchi *et al*. 2001, Wickstrom *et al*. 2002, Abdollahi *et al*. 2004, Wickstrom *et al*. 2004). Besides the C-terminal proteolytic fragments, there is also a soluble type XVIII collagen fragment derived from the NC11 domain, which contains the frizzled motif, with still unknown function (Elamaa *et al*. 2003). Most of the research on collagen XVIII was originally focused on endostatin because of its great potential to inhibit tumor angiogenesis. But it is also known that proteoglycans in the extracellular space play a major role in chemical signalling between cells as they can provide a reservoir for various secreted signalling molecules such as members of wingless/wnt, TGF-b and FGF families and cell adhesion molecule-like receptors (Lin *et al*. 2001, Aricescu *et al*. 2002). Therefore, collagen XVIII in the extracellular matrix may be important for defining plasticity of epithelial and endothelial cells in different organs and may be involved in determining cell polarity, influencing cell metabolism and differentiation (Lin *et al*. 2001).

Collagen XVIII is encoded by the *COL18A1* gene, mapped to 21q22.3. This gene has 43 exons, which are transcribed in 3 distinct isoforms by use of two promoters and of an alternative splicing of the third exon. The three proteins differ only by their signal peptides and by part of the N-terminal region of the NC11 domain, and contain 303 (NC11-303), 493 (NC11-493) and 728 (NC11-728) amino acid residues. NC11-303 mRNA is transcribed from the promoter located upstream of exon 1, and it does not contain exon 3. NC11-493 and NC11-728 mRNAs are both transcribed by the promoter located in intron 2 and do not include exons 1 and 2. Isoform NC11-728 and NC11-493 are encoded by the exon 3 and exons 4 through 43, but NC11-493 variant uses an internal splice donor site within exon 3, thus lacking 235 amino acid residues (Elamaa

et al. 2003). The NC11-493 variant mRNA is mainly expressed in fetal and adult liver, whereas the NC11-303 variant was found at the highest levels in the fetal kidney (Saarela *et al*. 1998). NC11-728,-is the only isoform containing the frizzled domain, it is also expressed in several tissues, with the highest levels in liver and lung (Elamaa *et al*. 2003). NC11-303 and NC11-728 are also expressed in human fetal brain and retina (Sertié *et al*. 2000, Suzuki *et al*. 2002). It seems that the expression pattern of the collagen XVIII isoforms change during development (Lin *et al*. 2001), suggesting that these molecules play important roles during development.

In 2000, we demonstrated that a mutation in the *COL18A1* gene causes an autosomal recessive disorder, Knobloch syndrome, showing for the first time the functional importance of the collagen XVIII molecule in humans (Sertié *et al*. 2000). We will focus this review on the distribution of pathogenic and polymorphic mutations along the *COL18A1* gene, their corresponding phenotypes and the possible functional roles of collagen XVIII based on the human and mice knockout phenotype.

Pathogenic Mutations and Knobloch Syndrome (KS)

Phenotype

Knobloch syndrome (KS; MIM 267750) is an autosomal recessive condition defined by the occurrence of high myopia, usually evidenced up to the first year of life, vitreoretinal degeneration with retinal detachment, macular abnormalities and occipital encaphalocele. Clinical variability is present but, to date, all patients have severe ocular abnormalities that usually lead to bilateral blindness. The occipital encephalocele is also a major clinical feature that has been described in 23 of 25 reported cases; its size varies among affected patients and sometimes it is noticed only through CT scan (reviewed in

Suzuki *et al*. 2002). Altered retinal pigmentation is a proeminent finding and more recently it was described a case of persistent fetal vasculature in the eye of a patient with KS (Seaver *et al*. 1993, Passos-Bueno *et al*. 1994, Kliemann *et al*. 2003, Duh *et al*. 2004).

Heterotopic nodules in the lateral ventricules and neuronal migration abnormalities in three KS patients have recently been noticed (Kliemann *et al*., 2003; Fernando Kok, personal communication). In two of these unrelated patients epilepsy was also present, suggesting that these clinical complication might be more common in KS patients than in the general population. In one patient, sagital craniosynostosis was also present; confirmation of this association in additional cases will expand the spectrum of clinical variability in KS.

Other minor clinical abnormalities, such as lens subluxation, cataracts, flat nasal bridge, midface hypoplasia, bilateral epicanthic folds, hypoplasia of the right lung with anomalous pulmonary return, cardiac dextroversion, generalizad hyperextensibility of the joints, unusual palmar crease and unilateral duplicated renal collecting system, have been observed in a few cases (reviewed in Suzuki *et al*. 2002). The spectrum of clinical variability of KS is still not defined since most of each of the above mentioned clinical alterations were reported only once in single families and before the identification of the gene.

Identification of Mutations in the *COL18A1* as Causative of KS

Based on the study of a single large inbred Brazilian family with 12 individuals affected with KS, we assigned the disease gene to 21q22.3 and demonstrated that a homozygous mutation at the AG consensus acceptor splice site of *COL18A1* intron 1 (IVS1-2A>T) causes Knobloch syndrome (Sertié *et al*. 2000). This mutation predicts

the skipping of exon 2 and creation of a stop codon in exon 4 and, therefore, the truncation of only the a1(XVIII) collagen NC11-303 (Sertié *et al*. 2000). Patients with this mutation present endostatin plasma levels within the normal range (Suzuki *et al*. 2002). This is expected as the isoform NC1-493, which produces most of the circulating endostatin, should not be altered by the presence of the IVS1-2A>T mutation (Sertié *et al*. 2000, Suzuki *et al*. 2002).

Pathogenic mutations in the *COL18A1* gene were found in 6 out of 9 other unrelated families with KS (3 Brazilian, 3 north-American and 3 European), thus confirming that mutations in this gene causes KS. A total of 7 different pathogenic changes (Table 1) were identified: three patients were compound heterozygotes and two homozygotes (Suzuki *et al*. 2002, unpublished data). Endostatin plasma levels, measured in 2 patients with null mutations, showed levels in the lower range, suggesting that these alterations lead to absence of the protein (Suzuki *et al*. 2002).

We did not identify mutations in three unrelated probands with the major typical clinical signs of the syndrome but, endostatin plasma levels, measured in two of them, showed values in the lower range (Suzuki *et al*. 2002, Kliemann *et al*. 2003). These data, therefore, suggest that the KS phenotype can be caused by an unusual mutational mechanism in *COL18A1* or to non-allelic genetic heterogeneity, as also suggested by others (Menzel *et al*. 2004).

We also did not find pathogenic mutations in KS patient who has high myopia associated with several other clinical features that were not typical of the syndrome: a scalp defect in the frontal region, developmental delay, telecanthus, hypertelorism, and high arched palate (Sniderman *et al*. 2000, Suzuki *et al*. 2002). This patient presented endostatin normal plasma levels. This finding suggests that this patient´s phenotype is probably caused by mutations at another locus, and that mutations in *COL18A1* cause

very specific ocular and neuronal cell migration abnormalities. Therefore, the minimum clinical features that define the diagnosis of KS are high myopia and occipital encephalocele.

Genotype-Phenotype Correlation in KS

All pathogenic mutations in the *COL18A1* gene to date probably lead to mRNA decay or to a truncated protein thus the disease might be caused by deficiency of a functional protein. So, these mutations represent human knockout models for the *COL18A1* gene: homozygotes for the IVS1-2T>A represent a knockout of only the short isoform, while the other mutations represent knockout of all collagen XVIII isoforms and endostatin.

Patients with deficiency of only the short isoform present the main clinical features of the syndrome, suggesting that the lack of this isoform is enough to cause the phenotype. It also seems that lack of only the short isoform is associated with a less severe ocular prognosis. These observations imply that collagen XVIII isoforms may play important functional roles in the human eye. The ocular alterations observed in KS patients are complex and, for obvious reasons, it is difficult to obtain a detailed histology and electron microscopic characterization of a KS patient´s eye. Thus, the histopathological and the molecular changes resulting from null *COL18A1* alleles in humans remain unknown and the study of mice models will be of great importance to elucidate them. Indeed, there has been a growing number of data suggesting that the Knockout mice of *col18a1*(*col18a1*-/-) constitute a promising model to study KS. These mice are viable and fertile, do not present occipital encephalocele but show abnormal visual function due to several ocular alterations which worsen with increasing age (Fukai *et al*. 2002, Marneros *et al*. 2003, Marneros *et al*. 2004). Developmental defects

in hyaloid vessel regression affecting postnatal levels of VEGF expression in the neural retina were observed in these mice (Fukai *et al*. 2002). Interestingly, Duh *et al*. (2004) described persistence of fetal vasculature in a patient with clinical features of Knobloch syndrome associated with no detectable plasma levels of endostatin. The iris basement membrane of the *col18a1*-/- mice was also shown to be altered (Marneros *et al*. 2003, 2004), further confirming that iris modifications in some KS patients is due to the deficiency of collagen XVIII (Passos-Bueno *et al*. 1994, Duh *et al*. 2004). Another important finding in the mice *col18a1*-/- is the age-dependent accumulation of electron-dense deposits between the retinal pigment epithelium (RPE) and Bruch´s membrane. These deposits contain excess basement membrane material and they were associated with an abnormal vitamin A metabolism in the retinal pigment epithelium with reduced rhodpsin levels (Marneros *et al*. 2004). These findings further confirm that endostatin/col18a1 plays a significant role in blood vessels remodeling and morphogenesis in the eye.

Variability of the size of the occipital alteration is commonly observed both in intra and interfamilial cases, but it does not have an obvious correlation with the site of the mutation in *COL18A1* gene.

Pathogenic mutations leading to deficiency of all collagen XVIII isoforms were found in two out of 3 KS patients who, besides minimum KS clinical features, also had abnormal neuronal cell migration and recurrent epilepsy. None of the 12 patients, all in middle age, with lack of only the short form of collagen XVIII has presented epilepsy; thus, we could speculate that the median and/or long collagen XVIII isoforms, in opposition to the short form, play an important role in neuronal cell migration. Alterations in neuronal cell migration have also been observed in col18a1 knockout animal models (Ackley *et al*. 2001). The current data, therefore, strongly support an

important functional role of collagen XVIII in neuronal development. It is possible that the occipital encephalocele actually represents abnormal cell migration instead of a defect in bone or suture development at the occipital region.

Col18a1-/- mice, depending on the genetic background, develop a significant enlargement of the skull associated with severe hydrocephalus. This clinical alteration presents incomplete penetrance and wide expressivity, with some mice presenting just enlargement of the ventricles visible only through MRI (Utriainen *et al*. 2004). This phenotype was not noticed in KS patients, maybe due to the small number of cases studied to date.

One of the patients (homozygous for the mutation c.2416 C>T; numbered according to the mRNA sequence AF018081, deposited in GenBank) has a duplicated kidney (data not reported). Lin *et al*. (2001) have shown that collagen XVIII expression is involved in kidney and lung morphogenesis, through still unknown mechanisms. Although no major kidney defect was observed among the mutant mice, they showed broadened kidney tubular basal membrane, altered mesangial matrix of the glomerulus and elevated serum creatinine levels (Utriainen *et al*. 2004). These findings further support the importance of collagen XVIII in kidney development and function. Therefore, KS patients should be carefully examined for functional and morphological kidney defects.

None of the patients with mutations in the *COL18A*1 gene have any major liver defect, which is surprising because one of the isoforms (NC11-493) is highly expressed in this organ.

Polymorphic Changes

We identified twenty polymorphic variants in the *COL18A1* gene (Iughetti *et al*. 2001, NCBI dbSNP database); of these, only two represent missense changes (I841V and D1437N). D1437N (previously referred as D104N) change is located in the COOH-terminal globular domain NC1 of collagen XVIII, the encoding region of endostatin. The aspartic acid at position 1437 (or amino acid residue 104 of endostatin) is conserved in human and mouse endostatin/collagen XVIII, as well as, in the endostatin-like molecule produced from the NC1 domain of collagen XV (Iughetti *et al*. 2001). The structural modeling analysis of the human mutated 1437N endostatin suggests that this amino acid residue is located at the surface of the molecule and does not change endostatin stability but can modify the charged distribution surrounding the mutated aminoacid. (Iughetti *et al*. 2001).

It has been postulated that endostatin/collagen XVIII play important roles in tumor growth due to its anti-angiogenic activity. Indeed, we have observed that Down syndrome (DS) patients, who have a decreased incidence of solid tumors, showed higher serum levels of endostatin (Zorick *et al*. 2001). Still others have shown that patients with a better prognosis of hepatocelular carcinoma presented higher collagen XVIII expression in tumor cells (Musso *et al*. 2001) and lower production of the molecule by the surrounding stroma (Hu *et al*. 2004). Therefore, endostatin and collagen XVIII are good candidate proteins to be studied in the susceptibility of tumor growth. In this regard, we have hypothesized that the SNP D1437N (or D104N/endostatin) could represent an at-risk susceptibility allele for progression or agressiveness of solid tumors. In a case-control study including 181 prostate cancer cases and 198 non-cancer individuals, we observed that the heterozygous N104 individuals have a 2.5 times increased chance of developing prostate cancer as

compared with homozygous D104 subjects (Iughetti *et al*. 2001). However, this association was not confirmed by us in another sample (data not published) as well as by others including different solid tumours (Ortega *et al*. 2003, Liu *et al*. 2003, Nascimento *et al*. 2004, Macpherson *et al*. 2004). It is possible that the variation of the genotypic frequencies observed in our original report just represent racial stratification. Further studies will be necessary to understand the role of collagen XVIII/endostatin and predisposition to solid tumors.

Conclusions

Collagen XVIII, encoded by the *COL18A1* gene at 21q22.3, is a very interesting molecule, which produces at least two proteolytic fragments: one derived from its C-terminal, endostatin and the other from its N-terminal, containing the frizzled motif. There are at least 3 distinct isoforms, which only differ at their N-terminal region; each of them present a specific tissue and development pattern of expression and possibly perform distinct functions.

Null alleles in the collagen XVIII lead to Knobloch Syndrome (KS), a rare autosomal recessive disorder mainly characterized by high myopia, vitreoretinal degeneration, macular alteration and occipital encephalocele. Iris and retinal pigment epithelium alteration seem also to be important features of the syndrome. The complete spectrum of the disease is still not established, but high myopia up to the first year of life and occipital encephalocele are the two minimal clinical criteria to classify a patient as KS. Collagen XVIII is a critical component of the basement membrane of the iris, vitreo and retina and its presence is fundamental for normal eye development during embryogenesis; this protein also seems to play important functional roles in neuronal

cell migration and as a component of basement membrane of kidney. Polymorphisms in collagen XVIII are common, but their relationship to susceptibility of solid tumors are still controversial.

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Table 1. Pathogenic mutations found in KS patients. Nucleotide numbers are based on the reference sequence AF018081.

Family	Pathogenic mutations detected
KS1	c.12-2A>T* (homozygous)
KS3	c.2969-2978delCAGGGCCCCC (maternal); c.3514-3515delCT (paternal)
KS4	c.1238-1239insA (maternal); c.3514-3515delCT (paternal)
KS5	c.3514-3515delCT (maternal); c.2105delC (paternal)
KS8	c.12-2A>T* (homozygous)
KS9	c.3277C>T (homozygous)
KS10	c.2416C>T (homozygous)

* mutation position based on the mRNA sequence AF018082.

Capítulo 6

CNS malformations in Knobloch syndrome with splice mutation in COL18A1 gene

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Abstract

Knobloch syndrome is a combination of posterior midline encephalocele, macular abnormalities, high grade myopia, vitreoretinal degeneration with retinal detachment and normal intelligence, suggesting alterations during early neuroectodermal morphogenesis. 28 cases have been reported insofar. It has been shown to be due to mutations in *COL18A1* gene, mapped to 21q22.3. It leads to abnormal expression of collagen XVIII and endostatin. Endostatin is the non triple-helical C-terminal NC1 globular domain of collagen XVIII. This proteolytically derived component is located in almost all epithelial basement membranes of capillaries and blood vessels, and in basement membranes of all major developing organs. Endostatin controls neuronal guidance in *Caenorhabditis elegans*. We report a 3 year-old girl born to consanguineous Maghrebian parents with high grade myopia (-15d), perimacular pseudo-colobomatous lesions, septo-optic dysplasia (septal agenesis and optic nerve hypoplasia), symmetric frontal micropolygyria, occipital meningocele and subnormal psychomotor development. This girl harbors a splice site mutation in intron 36, which is predicted to alter the splicing of the RNA. This patient expands the range of central nervous system (CNS) malformations observed with Knobloch syndrome and confirms the implication of tipe XVIII collagen/endostatin in neuronal migration and CNS formation.

Introduction

Knobloch syndrome (KS) is an autosomal recessive disorder associating severe myopia starting at a very early age and an occipital defect. The first description of the disease was made in 1971 in five affected sibs (Knobloch and Layer, 1972a; Cook and Knobloch, 1982). Fifteen families with a total of 39 affected patients have been reported so far, with more or less details (Czeizel *et al*., 1992; Seaver *et al*., 1993; Passos-Bueno *et al*., 1994; Wilson *et al*., 1998; Sniderman *et al*., 2000; Suzuki *et al*., 2002; Kliemann *et al*., 2003; Duh *et al*., 2004). In 1996, the gene responsible for Knobloch syndrome was mapped on the 21q22.3 locus in a large Brazilian family by linkage analysis (Sertie *et al*., 1996). In that family Sertie *et al*. (2000) found a mutation in the *COL18A1* gene coding for the collagen XVIII protein. Suzuki *et al*. (2002) tested 8 families and found mutations in 5 families confirming the involvement of *COL18A1*. Recently, Menzel *et al*. (2004) excluded linkage to the *COL18A1* locus in one family suggesting genetic heterogeneity in Knobloch syndrome.

Kliemann *et al*. (2003) reported the presence of an abnormal neuronal migration in two unrelated patients who showed heterotopic nodules at neuroimaging. One of them presented a localized pachygyria. We report here a mentally retarded girl with Knobloch syndrome associated with severe supratentorial CNS anomalies. This report confirms that neuronal migration can be affected in Knobloch syndrome and that it can be severe in some cases.

Clinical Report

This patient was the first child of healthy Algerian parents who were half first cousins, their fathers being half brothers. She has a younger brother in good health. Her mother was 27 years old at the time of her birth, and her father was 33. The mother had cystitis in the first 3 months of her pregnancy. She was treated with amoxicillin and nitroxolin, without any complications. The ultrasonographic investigations during late pregnancy term showed microcephaly and excessive femoral length.

Birth was uneventful except for a transient tachypnea which improved spontaneously after one day. Birth weight was 2940 g and birth length was 56 cm. Head circumference at birth is unknown. At birth, an occipital meningocele was noted. There were no cerebellar structures in the pouch which initially had a diameter of 5 cm and expanded progressively. The meningocele was removed surgically at the age of 8 months. She had no seizures. Nystagmus was noted at the age of 6 months. She held her head at the age of 9 months and was able to stand at 12 months. When examined at the age of 24 months, her height was 91 cm (+2SD), her weight was 14 kg (+2SD) and her head circumference was 50 cm (+1SD). She was able to say a few words and had difficulties in understanding simple orders. The patient showed mild facial dysmorphism: narrow face, high and large forehead, horizontal eyebrows, bilateral epicanthic folds, bulbous nasal tip and temporal narrowing. Neurologic examination and electroencephalogram (EEG) were normal. Ophthalmologic examination showed horizontal nystagmus, severe, rapidly worsening myopia : -15.25 (-1.75x150) OD and -15.75 (-1.25x175) OS with best corrected visual acuity of 20/200 J3 OU. The ocular axial length was 27.81 mm OD and 28.18 mm OS by ultrasound. Anterior segment was normal. Fundus examination revealed a severe myopic retinopathy. Early signs of

vitreous degeneration were noted. Unusual round-shaped chorioretinal pseudo-colobomatous lesions were present on both sides in the inferior temporal retina, extending to the paramacular area. No retinal pigment was visible in the lesions or their surroundings. Electroretinogram (ERG) and visual evoked potential (VEP) explorations were normal. Besides an occipital bone defect resulting from surgery, cranial X rays and MRI neuroimaging showed agenesis of the septum pellucidum, frontal pachygyria/polymicrogyria, and heterotopic hypersignals (on TW2 images) along the radial migration tracts. Psychometric evaluation at the age of 3 years and 11 months was complicated by visual impairment and distractibility. Using Brunet-Lezine and Vineland scoring system, and some tests of the WPPSI-III chart, a developmental level between 2 years 2 months and 3 years 6 months was evaluated, corresponding to a QD of 75.

Molecular Investigations

All the coding regions of the gene, including the exon-intron splice junctions, were PCR amplified and sequenced in an automatic sequencer (MegaBACE, GE Healthcare) for mutation screening. We identified a single nucleotide change located near the donor splice site of intron 36, present in homozygosity in the patient. This mutation, c.3544+3A>C (numbering according to the sequence AF018081 deposited in GenBank), was not detected in a 100-chromosome control sample and this nucleotide is conserved in mice and humans. Considering the fact that the third nucleotide of an intron is usually a purine (Stephens and Schneider, 1992) and this position is conserved between different species, this change is likely to be pathogenic and it is possibly critical for the processing of the RNA.

Discussion

A diagnosis of Knobloch syndrome (KS) in our patient is sustained by the combination of high grade myopia due to excessive increasing of ocular axial length and midline skull defect, it is confirmed by the presence of the mutation c.3544+3A>C in the *COL18A1* gene. Ocular anomalies are a major feature in KS, usually with a poor prognosis and usually resulting in complete loss of vision that can occur at different ages (Passos-Bueno *et al*., 2006). Most patients need an early correction of –10 diopters or higher. As in this report, vitreoretinal degeneration, a non specific consequence of severe myopia, is almost always present in the form of vitreous liquefaction and pigmentary lesions. Retinal detachment is the main complication and occurs before the age of 15 in most cases, preventive cryotherapy is often inefficient. Lens opacities are also frequently found in KS and they can develop into totally opaque lenses. Our patient did not show these lesions at the age of 5 years. Her fundi showed an unusual paramacular retinal coloboma. This lesion has not been associated with any particular disorder (Pian *et al*., 2003). The defect is different from the retinal aspect of Aicardi syndrome since there is no pigmentation either inside the lesion or in its surroundings. Kliemann *et al*. (2003) also reported a retinal coloboma in a patient with KS; as the lesion is not precisely described, we ignore if the unusual appearance of the defect is really distinctive.

Midline scalp defect is the second most frequent sign of KS. The defect is always in the occipital midline region; this hypothesis has been further supported by the observation that a patient with a frontal defect and clinically classified as KS did not present mutations in the *COL18A1* (Sniderman *et al*., 2000). It should be stressed that five patients have been described without this feature (Knobloch and Layer, 1972;

Wilson *et al*., 1998; Kliemann *et al*., 2003), and in those cases the diagnosis was possible only because of the presence of the complete phenotype of the syndrome in a sib. We can therefore suppose that sporadic KS can be underdiagnosed in patients presenting only severe myopia. The nature of this defect is still controversial, in most cases, the evaginated pouch do not seem to contain brain tissue at neuroimaging or macroscopic examination, although histological examination showed heterotopic, poorly formed neural tissue in some cases (Czeizel *et al*., 1992; Seaver *et al*., 1993; Wilson *et al*., 1998). For Wilson *et al*. (1998), such findings characterize the scalp defects in KS as encephaloceles rather than meningoceles. In our patient, the scalp lesion was macroscopically described as a meningocele, but unfortunately no histological examination was made.

Till 2003, supratentorial CNS and development were considered to be normal in KS. Recently Kliemann *et al*. (2003e) reported brain anomalies in 2 unrelated patients with KS. Both showed a neuronal migration disorder with heterotopic nodules scattered in the white matter. One of them had a unilateral area of pachygyria in the frontal lobe. These patients did not show any neurological sign, and their psychomotor development was normal. Our patient gives an independent confirmation that neuronal migration is affected in KS, as she presents heterotopias, bilateral pachygyria covering all the frontal lobes. Agenesis of the septum pellucidum has not been described before in KS. Usually this lesion is associated with septo-optic dysplasia (most often) or holoprosencephaly (Malinger *et al*., 2005). In our patient, there were no signs of holoprosencephaly (even mild) and the optic nerve papillas were normal. Not surprisingly, our patient had borderline mental delay and learning disability, another feature that has not been reported so far in association with KS.

The *COL18A1* gene has at least 3 variant transcripts. The longest transcript is expressed in the liver, lung, skeletal muscle, spleen, thymus and kidney; the medium transcript is expressed almost exclusively in the liver, while the shorter variant is mainly expressed in kidney but it is also found in most of the other tissues of the body, including retina (Saarela *et al*., 1998; Sertie *et al*., 2000; Suzuki *et al*., 2002; Elamaa *et al*., 2003). Surprisingly, no hepatic or kidney lesion has ever been reported in KS patients. In addition, endostatin, a proteolytic cleavage product of the C-terminal part of collagen XVIII, is a potent inhibitor of angiogenesis (O'Reilly *et al*., 1997). All KS patients with CNS abnormalities, including the case here reported, bear mutations that lead to deficiency of all the 3 collagen XVIII isoforms. Considering the fact that the first patients reported as carriers of *COL18A1* mutations, who lack only the shortest collagen isoform and were older than 40 years of age did not present neuronal migration abnormalities (Sertie *et al*., 2000), we could speculate that major CNS abnormalities are related to deficiency of the medium and/or large isoforms or endostatin.

In conclusion, this report further contributes to a better characterization of the ocular alterations due to deficiency of collagen XVIII and adds new data on the effects of the deficiency of this collagen in CNS. Further reports are still needed to elucidate whether CNS involvement depends on specific mutations, and to evaluate the risk of recurrence of mental handicap. Both issues are of major importance for genetic counseling.

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Capítulo 7

Functional analysis of endostatins T48 and N104 and skin biopsies as a diagnostic tool for Knobloch Syndrome.

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Abstract

Endostatin, a proteolytic cleavage product of the C-terminal domain of type XVIII collagen, is a potent angiogenesis inhibitor that influences endothelial cell proliferation, migration, apoptosis and tubulogenesis. Endostatin is a molecule that binds to several extracellular matrix (ECM) components, including laminin-1, fibulin-1, fibulin-2, nidogen-2, perlecan, heparan sulfate and fibronectin. Null mutations in *COL18A1* (MIM ID 120328) cause Knobloch syndrome (KNO; MIM ID #267750), an autosomal recessive condition characterized by high myopia, macular abnormalities, vitreoretinal degeneration, retinal detachment and occipital encephalocele. It has been suggested that a polymorphic residue change (p.Asp1437Asn; D104N) in endostatin predisposes its carriers to the development of prostate cancer but this proposition has not been confirmed. Another rare residue change in the endostatin domain (p.Ala1381Thr; A48T) was found in two Knobloch Syndrome (KNO) patients but its relation to the phenotypic characteristics seen in these patients is still unknown. In order to evaluate the effect of these variations in endostatin, we have produced recombinant human N104 and T48 proteins and determined their binding to other ECM proteins using ELISA and a surface plasmon resonance method. In addition, we have performed for the first time collagen XVIII (c.18) immunofluorescent histochemical analysis of skin biopsies from 4 patients with KNO, and show that collagen XVIII is indeed absent, thus confirming that the disease is caused by absence of this collagen. Our results suggest that the mutation A48T, but not D104N, might alter the functional activity of endostatin, but it is still not possible to classify this mutation as pathogenic. We also suggest that analysis of collagen type XVIII in small skin biopsies using an immunofluorescence method is a reliable test, which could allow the screening of a larger number of patients with a possible diagnosis of KNO.

Introduction

Angiogenesis is a process involved in numerous diseases such as cancer, rheumatoid arthritis and diabetic retinopathy. It is believed to be regulated by a balance of proangiogenic (e.g., VEGF, TGF-α) and antiangiogenic factors (e.g., endostatin, tumstatin, thrombospondin-1) (Hanahan and Folkman, 1996). Endostatin is a proteolytic cleavage product of the C-terminal domain of type XVIII collagen which appears to function as a potent angiogenesis inhibitor that influences endothelial cell proliferation, migration, apoptosis and tubulogenesis (Dhanabal, et al., 1999; Ergun, et al., 2001; O'Reilly, et al., 1997; Yamaguchi, et al., 1999). The different responses observed in endothelial cells in the presence of endostatin appear to result from the activation of distinct signaling pathways that completely change the expression profile of the cells (Abdollahi, et al., 2004). The mechanisms of action underlying these effects are not completely known. Endostatin is an adhesion molecule that binds to several extracellular matrix (ECM) components, including laminin-1, fibulin-1, fibulin-2, nidogen-2, perlecan, heparan sulfate and fibronectin (Hohenester, et al., 1998; Sasaki, et al., 1998; Sasaki, et al., 2000). The only known cell surface ligands are $\alpha_5\beta_1$ integrin and glypican (Karumanchi, et al., 2001; Rehn, et al., 2001).

The trimeric type XVIII collagen molecule is a basement membrane component encoded by the *COL18A1* gene (Oh, et al., 1994; Rehn, et al., 1994). Although type XVIII collagen is localized in most basement membranes (Saarela, et al., 1998), its function is not well understood. The endostatin fragments derived from type XVIII collagen in the basement membrane may, however, function along with other antiangiogenic factors, to prevent blood vessels from penetrating the basement membrane and entering the overlying, normally avascular, epithelial layers. The lack of

type XVIII collagen has been identified as the cause of Knobloch syndrome (KNO), an autosomal recessive condition characterized by high myopia, macular abnormalities, vitreoretinal degeneration, retinal detachment and occipital encephalocele (Cohen and Lemire, 1982; Passos-Bueno, et al., 1994; Sertie, et al., 2000; Suzuki, et al., 2002).

The polymorphism p.Asp1437Asn located in the endostatin domain of type XVIII collagen (D104N; residue 104 of the endostatin domain) has been found in heterozygosity or homozygosity in healthy people and KNO patients with an allelic frequency of about 10% in European derived individuals. It is still unclear whether this polymorphic change has an effect on endostatin function or if it is involved in the development of tumors or other pathogenesis (Iughetti, et al., 2001; Menzel, et al., 2004).

The p.Ala1381Thr variation is another change present in the endostatin domain (A48T; residue 48 of the endostatin domain) that was found in homozygosity in one consanguineous KNO family with two affected children who display a typical KNO phenotype (Kliemann, et al., 2003). The base substitution (c.4141G>A: gi:2920534) that leads to a residue change in a highly conserved site in the protein, was the only mutation found in the coding region of *COL18A1* in this family. Recently, Stahl et al. (2005) produced a recombinant human T48 endostatin using a mammalian expression system and reported a normal *in situ* binding despite its apparent reduced folding efficiency. In the present work we evaluated the *in vivo* and *in vitro* characteristics of these two naturally occurring missense changes in endostatin. We assessed the presence of type XVIII collagen in skin biopsies of two Knobloch syndrome patients carrying the A48T change and two patients carrying the nonsense mutation p.Gln1273X (c.3817C>T: gi:2920534). We also tested the binding properties of recombinant N104 and T48 endostatins to heparin, laminin, nidogen-1, type IV collagen, fibulin-1, laminin-1-

nidogen-1 complex and perlecan in order to verify if these changes alter endostatin / type XVIII collagen function at the molecular level.

Materials and Methods

Skin Biopsies

Skin punch biopsies (5 mm) were obtained from the forearms of 4 KNO patients belonging to two unrelated families. Clinical and molecular analyses of these patients were described in detail by Suzuki et al. (2002) and Kliemann et al. (2003). Control skin samples were obtained from normal patients undergoing surgery. This project was approved by the Ethical Committee of the Institute of Biosciences, USP and tissues were collected only after receiving informed consent.

Tissue samples were fixed according to procedure described elsewhere (Carvalhaes et al., 2006). Briefly, samples were immediately fixed and cryosubstituted in a -70°C solution of 80% methanol/20% dimethyl sulfoxide for 5-7 days, transferred to -20°C for 1-2 days, then brought to room temperature, rinsed 3x in absolute ethanol and embedded in Paraplast Plus following standard protocols. In addition to maintaining morphological structure, this method also appears to preserve the antigenicity to a higher degree than aldehyde fixatives.

Immunofluorescent (IF) Histochemistry

Seven μm thick sections were dewaxed with xylene and rehydrated through a graded series of ethanol into phosphate-buffered saline (PBS). Testicular hyaluronidase (1 mg/ml; Sigma) in 50 mM acetate buffer, pH 5, was added at room temperature for 30 min and followed by several washes in PBS. Blocking was achieved using 2% bovine

serum albumin (BSA) in PBS at room temperature for 1 hr followed by an overnight incubation at 4°C with primary antibodies diluted in 0.1% BSA/0.01% Tween 20 in PBS. A rabbit polyclonal antibody against type XVIII collagen (QH48.18, "anti-all") which recognizes all three isoforms of type XVIII collagen (Saarela et al., 1998) was used at a dilution of 1:300. An antibody against type IV collagen (rabbit anti-human type IV collagen polyclonal antibody, diluted 1:400; Rockland) was used as a basement membrane marker. After several rinses in PBS, sections were incubated for 1 hour at room temperature with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:300; Jackson Laboratory). After several rinses in PBS, sections were mounted in 10% Tris-HCl, pH 9.0/90% glycerol and analyzed using a laser scanning confocal microscope (Zeiss 510META).

The distribution patterns and levels of expression of types IV and XVIII collagens were analyzed using pseudocolor images made with the thermo lookup table in the Confocal Assistant 4.02 freeware program (http://www.cyto.purdue.edu/flowcyt/software/cas402.htm). This tool utilizes grey-scale pixel values to generate pseudocolor images that allow the discrimination of small differences in the level of immunofluorescence: i.e., lighter, higher pixel values are artificially colored with warmer, red colors which indicate increased immunostaining, whereas areas with decreased antigen levels are darker, have lower pixel values, and are displayed as cooler, blue to black colors.

Molecular Modeling

Human endostatin structure (1BNL) was obtained from the Protein Data Bank (http://www.rcsb.org), the A48T change was created and analyzed using GRASP (Nicholls, et al., 1991) and Insight-II/Discover software.

Recombinant Endostatin Production

The coding region of the endostatin domain was cloned into the pET-15b vector (Novagen, Madison, WI). Endostatin production and purification were based on a procedure published elsewhere (Rehn, et al., 2001). N104 and T48 endostatin were produced by PCR-based site directed mutagenesis.

Recombinant human endostatin was produced in *E. coli* as described previously (Rehn, et al., 2001) with slight modifications. Briefly, endostatin expression was induced using 1mM IPTG for 3 hours, cells were isolated by centrifugation and lysed by freeze thaw cycle in GUMCAC-0-Buffer (6M guanidine-HCl, 0.5M NaCl, 20 mM Tris-HCl, pH 7.9) followed by sonication in the presence of 15 mM β-mercaptoethanol. Lysate was loaded onto a ProBond™ column (Invitrogen) pre-equilibrated with URMCAC-0 buffer (8M urea, 0.5 NaCl, 20 mM Tris-HCl, pH 7.9). Endostatin was eluted with a gradient concentrarion of imidazole (0 to 500 mM) and dialyzed against 4 M urea, 0.1 M NaCl, 1 mM reduced glutathione, 0.1 mM oxidized glutathione, 20 mM Tris-HCl pH 7.9 (16 hours at 4°C), followed by a dialysis against 1 M urea, 0.1 M NaCl, 0.1 mM reduced glutathione, 0.01 mM oxidized glutathione, 20 mM Tris-HCl pH 7.9 (6 hours at 4°C) and a final dialysis step against PBS pH 6.9 (16 hours at 4°C). Human endostatin was then loaded onto a HiTrap-SP column (Amersham) and eluted using a NaCl gradient (0 to 1.5 M) in PBS pH 6.9. Buffer was exchanged by dialysis against 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4 for 16 hours at 4°C.

Binding Assays

Surface plasmon resonance (SPR) assays were performed with a BIAcore® 3000 instrument (Biacore AB, Uppsala, Sweden). Extracellular matrix proteins, the

laminin-1-nidogen-1 complex, perlecan, and fibulin were immobilized on CM5 sensor chips (Biacore AB) as described previously (Tu, et al., 2002). Mouse laminin-1-nidogen-1 complex and mouse perlecan were prepared from the mouse Engelbreth-Holm-Swarm tumor (Timpl, et al., 1987). The microfibrillar component fibulin-1 was prepared by recombinant production (Sasaki, et al., 1995). Binding assays were carried out in triplicates in 0.02 M Tris-HCl, 0.11 M NaCl containing 0.05% P-20 surfactant (Biacore AB) at a flow rate of 20 µl/min. The association phase was monitored for 3 min, and the dissociation curve was recorded for 10 min. The bulk effects were substracted using the reference control surfaces. The chips were regenerated by treatment with 1 M NaCl for 30 sec. For the calculation of kinetics constants, the sensorgrams at concentrations of 0, 10, 50, 200, and 400 nM were fitted globally to the 1:1 Langmuir model with BIAevaluation software version 3.1.

ELISA assays were based on a procedure used by Rehn et al. (2001). Laminin, type IV collagen, nidogen-1, fibulin-1 and heparin-BSA (10 µg/ml) were coated onto the surface of microtiter wells at 4 °C overnight. All the other steps were performed at room temperature. The wells were blocked with 5% non-fat milk in 0.05 M Tris-HCl, pH 7.4, 0.11 M NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ (TBS-Ca/Mg) for 1 h and then washed with 0.05% Tween 20 in TBS-Ca/Mg, and incubated for 3 h with endostatin as a soluble ligand diluted in 5% non-fat milk TBS-Ca/Mg. After thoroughly washing, the samples were incubated for 1 h with the antibody HES.6 against the human endostatin domain (Heljasvaara, et al., 2005) diluted in TBS-Ca/Mg-5% milk, followed by washing with 0.05% Tween 20 in TBS-Ca/Mg, and incubation with the secondary antibody conjugated with horseradish peroxidase. Endostatin bound to the immobilized proteins was detected by adding 5-aminosalicylic acid (Sigma) in the presence of 0.01% H_2O_2. The detection was performed at 490 nm.

Results

Fluorescent immuhistochemical analysis of skin biopsies from two individuals with KNO carrying the change A48T and two other individuals carrying the nonsense mutation p.Gln1273X demonstrated a complete lack of expression of type XVIII collagen anywhere in the tissue sections, while control samples displayed a high level of staining in the basement membranes of the epithelial layer and blood vessels (Fig. 1). The lack of expression of type XVIII collagen was especially evident in pseudocolor, thermo images in which even minor amounts of immunofluorescently labeled material can be detected. Although another basement membrane protein, type IV collagen, showed a similar distribution pattern in both control and KNO groups, its immunostaining intensity in KNO samples was noticeably lower.

We have previously reported the modeling of N104 endostatin and predicted a change in the electrostatic potential of the protein that could interfere with its activity by affecting the interaction with other proteins (Iughetti, et al., 2001). However, the ELISA and SPR assays performed in this study did not reveal a great difference between N104 and wild type endostatin. Binding to heparin-BSA and laminin were stronger, while both endostatins bound weakly to all other immobilized ligands, even after incubations with high endostatin concentrations. In SPR experiments, the laminin-1-nidogen-1 complex, fibulin-1, and perlecan were immobilized onto the CM5 sensor chips, while wild type and mutant endostatin were analyzed as soluble analytes (Fig. 2). N104 endostatin showed affinities to these molecules comparable to the wild type protein (Table I).

Our molecular modeling analysis of the T48 endostatin suggests that it is possible to accommodate the threonine side chain in this position (Fig. 3). ELISA assays showed

the binding properties of the T48 endostatin to be very close to the wild type endostatin, without any significant differences between them. In contrast to N104, however, SPR analysis revealed that the T48 mutation leads to a somewhat weaker binding affinity to some of the ECM proteins that were tested (Table I). The differences between wild type and T48 endostatin binding to laminin-1-nidogen-1 complex and to fibulin-1 were small but significant (P=0.0017 and P=0.0011, respectively). Perlecan binding was weak for all endostatins and the differences were non-significant.

Discussion

This paper presents the first immunohistochemical evaluation of type XVIII collagen in KNO patients. We have analyzed the distribution pattern of type XVIII collagen in skin biopsies of 4 KNO patients: two sibs with the known pathogenic nonsense mutation p.Gln1273X and two other sibs with the missense endostatin mutation A48T. As shown in figure 1, we were unable to detect type XVIII collagen in any of the KNO patients tested as compared to controls, thus demonstrating that the disease is caused by deficiency of this collagen. Interestingly, the KNO samples also show a lower expression level of type IV collagen when compared to the control samples. Since endostatin/collagen XVIII binds to several ECM components as shown here and elsewhere (Hohenester, et al., 1998; Sasaki, et al., 1998; Sasaki, et al., 2000), it is possible that a lack of this protein could generate changes in the overall organization and stability of the ECM, especially in the basement membrane where it is normally expressed.

We have previously shown that endostatin serum levels measured by an ELISA assay were lower in KNO patients as compared to controls (Suzuki, et al., 2002). Although we were able do discriminate patients and controls through this method, we

raised the hypothesis that the antibody used in the assay presents cross-reactivity. This is evidenced by the positive endostatin levels in KNO patients that are carriers of null mutations, which should lead to a total or nearly total deficiency of type XVIII collagen. The immunohistochemistry results confirm our prediction that null mutations in the *COL18A1* gene lead to a severe deficiency of the protein. Therefore, immunohistochemical assessment of type XVIII collagen for diagnosing KNO may be more precise than endostatin measurements through ELISA assays. It also allows for a type XVIII collagen deficiency screening on a larger set of suspected patients with mutations in this gene. The mutation screening of *COL18A1* gene is still expensive and very laborious and the effect of some detected mutations can be uncertain, as is the case of the endostatin A48T change. Even when a possible pathogenic mutation is found, the immunohistochemical analysis of skin biopsies can be an important tool for the confirmation of the diagnosis.

Based on our results, the D104N change in endostatin does not appear to significantly affect its binding to the tested molecules, which is in disagreement with the results obtained by Menzel et al. (2004), who reported that the N104 endostatin has a weaker binding affinity to laminin by using immunoprecipitation. However, it is still possible that this residue impairs the interaction with other proteins. There is growing evidence against the hypothesis that this change is functional, as the association results between the N104 endostatin and prostate cancer found by Iughetti et al. (2001) have not been reproduced in other studies (Li, et al., 2005; Liu, et al., 2003; Macpherson, et al., 2004; Nascimento, et al., 2004; Ortega, et al., 2003; Passos-Bueno, et al., 2006). In addition, Stahl et al. (2005) most recently also showed that recombinant N104 endostatin presents normal binding to all basement membranes. Considering all this

data, it seems unlikely that the D104N change results in significant functional alterations.

In contrast to the N104, the T48 endostatin showed a somewhat weaker binding affinity to the tested ECM proteins, even though the molecular modeling of the protein did not predict a great modification. It is interesting to note that molecular modeling does not have a good predictive power on the effect of residue substitutions such as this. Indeed, Stahl et al. (2005) using a recombinant human T48 endostatin in a mammalian expression system suggested that this change lead to an apparent reduced folding efficiency. These authors also observed a reduction by 40 % in T48 endostatin production. However, considering that KNO is caused by a complete lack of type XVIII collagen, the current data does not allow us to conclude that this mutation leads to the KNO phenotype. There is still a possibility that the KNO in this family is caused by an undetected mutation, since there was no detectable type XVIII collagen. Finally, we are showing for the first time the possibility to diagnose KNO through the analysis of the protein, which could allow a screening of a larger number of patients with a possible diagnosis of KNO.

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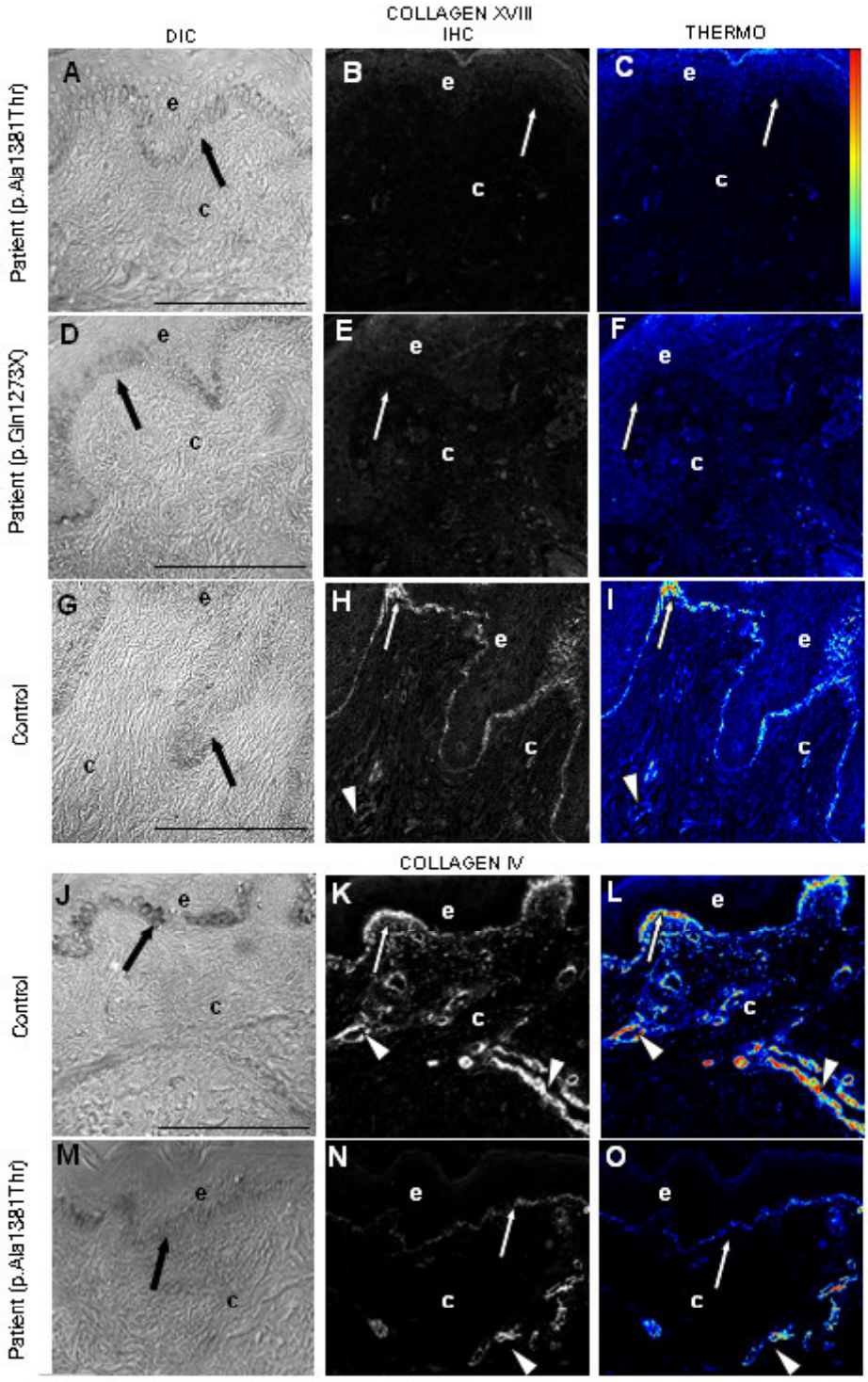

Figure 1. Immunofluorescent localization of type XVIII collagen in KNO and control skin. Differential interference contrast (DIC) images (left panels) show that the epithelial and connective tissues obtained from skin biopsies are intact and well preserved using the cryofixation and cryosubstitution techniques. Immunolocalization analysis of KNO carrying the p.Ala1381Thr (A48T) change (A-C) or the nonsense mutation p.Gln1273X (D-F) showed a negative expression for collagen XVIII as compared to controls (G-I). Pseudocolor, thermo images (right panels) showed that collagen XVIII expression was present in control samples but undetectable in KNO samples. Although the distribution pattern of type IV collagen was similar in both control (J-L) and KNO (M-O) groups, the immunostaining intensity in KNO samples was noticeably lower. The color bar to left of panel C indicates immunofluorescence staining intensity: red, higher levels; blue/back, lower levels. Arrows, basement membrane; arrowhead, blood vessels. Scale bars: 100mm.

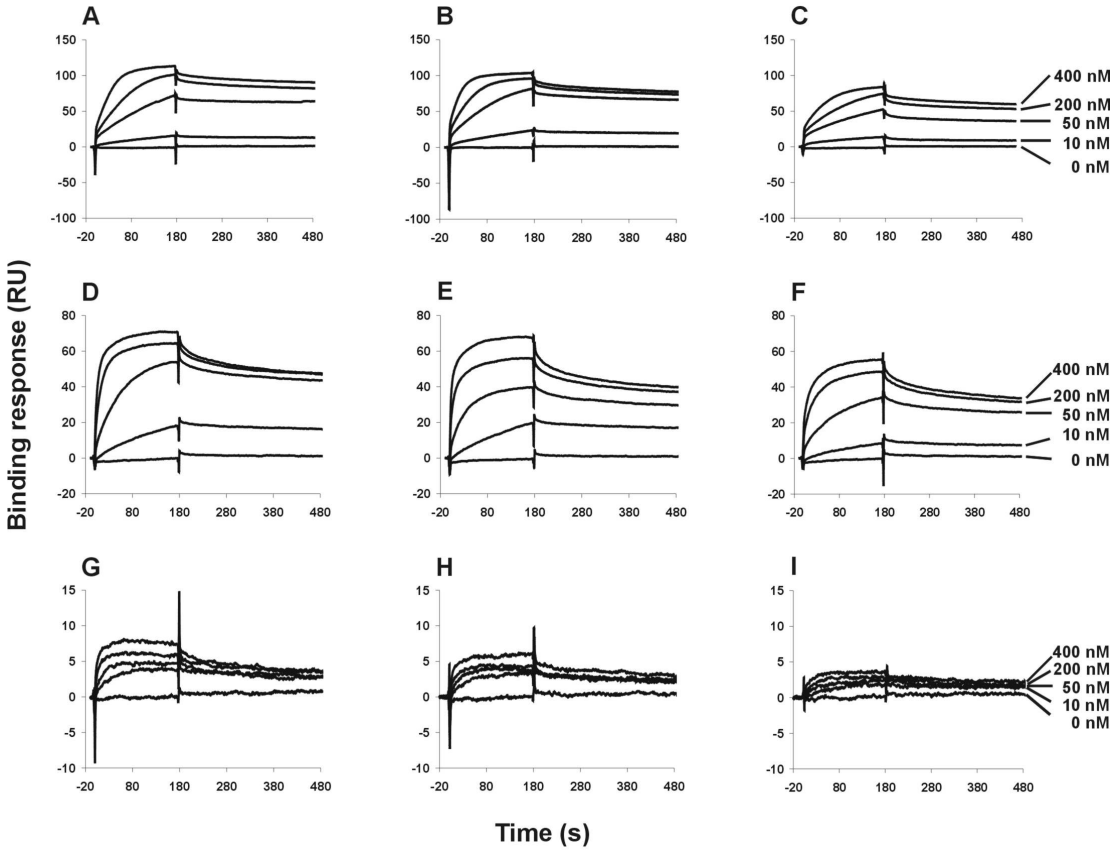

Figure 2. Interaction of recombinant endostatin with laminin-1-nidogen-1 complex, fibulin, and perlecan in a surface plasmon resonance assay. Endostatin variants (wild type, mutant N104, mutant T48) were diluted to a concentration series in 0.02 M Tris-HCl, 0.11 M NaCl containing 0.05% P-20, and then injected into the sensor chips immobilized with laminin-1-nidogen-1 complex (A-C; A=wt, B=N104, C=T48), fibulin (D-F; D=wt, E=N104, F=T48), and perlecan (G-I; G=wt, H=N104, I=T48) at 25ºC with a flow rate of 20 µl/min. Sensorgrams show binding of various concentrations of endostatin to the coated sensor surfaces. The association curves were monitored for 3 min, and the dissociation phases were recorded for 10 min but presented for 5 min. All the kinetics studies were performed three times independently at concentrations of 0-400 nM, and the data were analyzed with BIAevaluation software version 3.1 using the 1:1 Langmuir binding model.

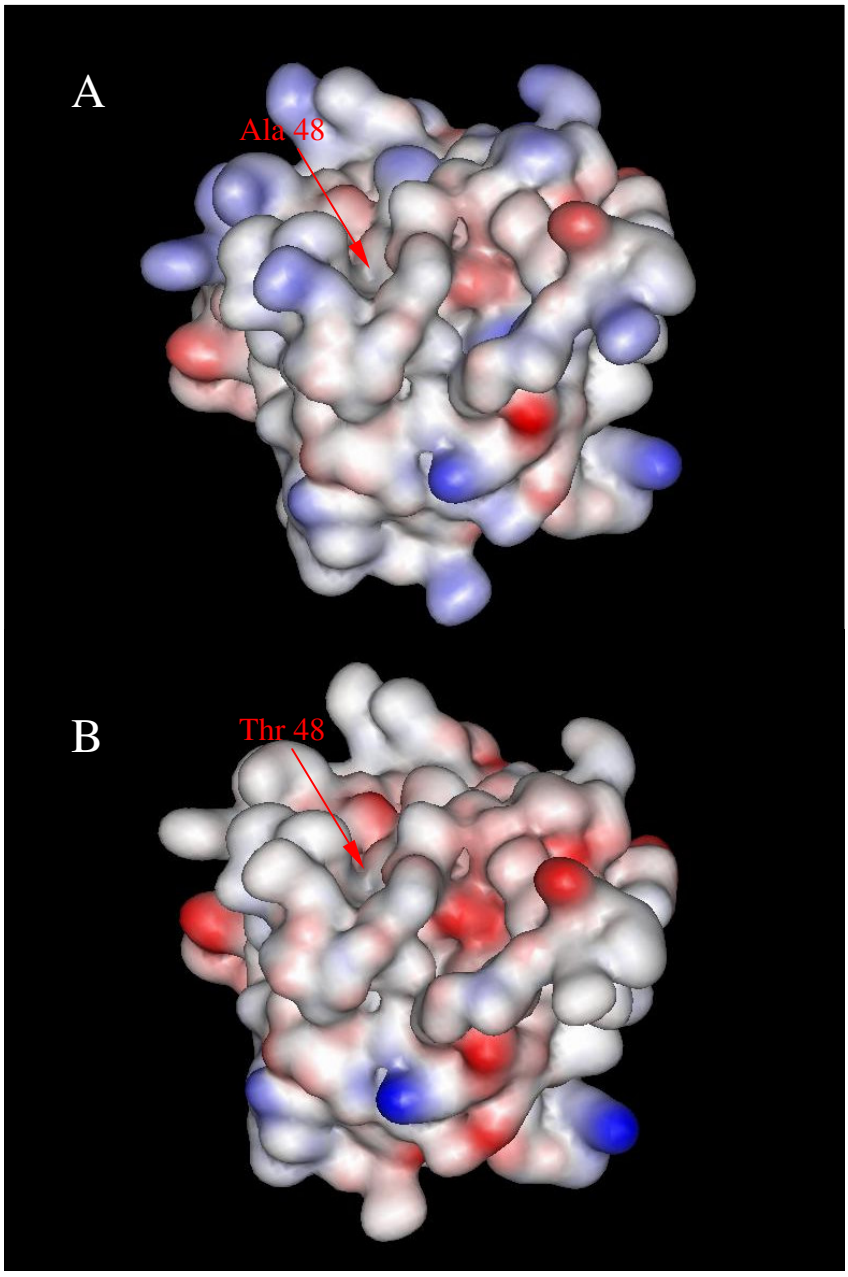

Figure 3. Molecular modeling analysis of the electrostatic surface of A48 (A) and T48 (B) endostatins. Areas shown in red represent a negative potential while the blue areas are positive.

Table I. Binding affinities (K_D) of wild type and mutant endostatins to laminin-1-nidogen-1 complex, fibulin-1, and perlecan

	laminin-1-nidogen-1 complex	Fibulin	perlecan
Wild type	3.48±0.24 nM	3.10±0.39 nM	5.53±1.53 nM
N104	2.98±0.28 nM	2.38±1.05 nM	4.34±2.13 nM
T48	6.17±0.27 nM	6.95±0.25 nM	5.19±0.92 nM

Interação Do Domínio Trombospondina Com Outras Proteínas

Introdução

Na região do domínio NC11 compartilhada entre as três isoformas conhecidas do colágeno tipo XVIII, é encontrado um segmento (possuindo aproximadamente 200 resíduos de aminoácido codificados pelo exon 4) homólogo à trombospondina (Rehn *et al*., 1994; Saarela *et al*., 1998a). Este domínio encontra-se conservado entre os colágenos XVIII ortólogos de outros organismos. A trombospondina é uma glicoproteína secretada com afinidade por diversas moléculas e que apresenta atividade antiangiogênica (Bornstein, 2001). No entanto, não se sabe ainda qual o significado funcional dessa seqüência homóloga à trombospondina no colágeno XVIII, bem como nos colágenos V, IX, XI, XII e XIV, nos quais também é observada. Os resíduos da trombospondina conhecidamente envolvidos em interações com heparina não estão conservados neste domínio do colágeno XVIII (Rehn *et al*., 1994).

Uma das formas de se caracterizar funcionalmente uma seqüência polipeptídica é a identificação de proteínas que interajam com ela, estabelecendo-se desta forma as vias bioquímicas, complexos ou redes de interação em que a proteína se encontra. O sistema de duplo-híbrido vem sendo amplamente empregado na identificação de interações desconhecidas, utilizando-se uma proteína de interesse como isca na triagem de bibliotecas de cDNA.

Apresentamos aqui a utilização de um sistema de duplo-híbrido para a identificação de possíveis ligantes para o domínio trombospondina do colágeno tipo XVIII murino.

Material e Métodos

O ensaio de duplo-híbrido foi realizado utilizando-se o sistema ProQuest ™ (Invitrogen) com a utilização da biblioteca de cDNA total de embrião de camundongo (E10,5) clonada em vetor pPC86 (Invitrtogen). Os vetores pDBLeu e pPC86 e as linhagens MaV203, Contrtoles A, B, C, D e E de levedura foram gentilmente cedidos pelo Prof. McInnes (Universidade de Toronto, Departamento de Genética Médica) para nosso estudo.

Para a obtenção da região codificadora do domínio trombospondina do colágeno tipo XVIII murino, realizamos a amplificação do cDNA de fígado de camundongo. A região inserida no vetor pDBLeu foi selecionada com base no alinhamento entre a seqüência da proteína humana (gi:18765747; NCBI http://www.ncbi.nlm.nih.gov), do camundongo (gi:40789281) e a seqüência consenso do domínio trombospondina (número de acesso PF02210; http://www.sanger.ac.uk/Software/Pfam), todos encontrados em banco de dados (Figura 1).

Os oligonucleotídeos utilizados para a amplificação foram os seguintes: Thromb mouse F (5'-CTCGAGTGAGGTGGGGCTGCTGCAGCTC-3') e Thromb mouse R (5'- GCGGCCGCAGACAGTGCACAGGGCTCAC-3'). O produto da amplificação foi ligado no vetor pDBLeu com a utilização das enzimas *Xho*I e *Not*I e transformado em *Escherichia coli*.

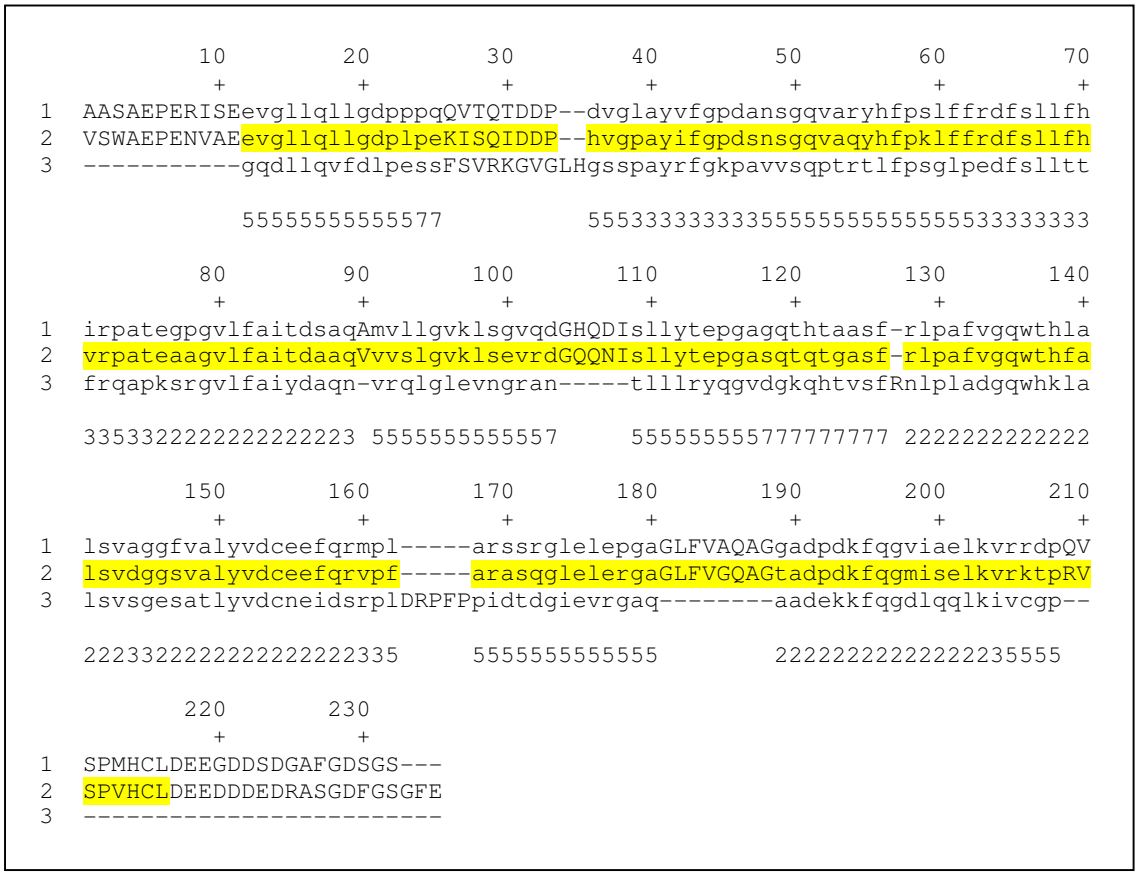

Figura 1. Alinhamento múltiplo da região do domínio trombospondina do colágeno tipo XVIII humano (seqüência 1), murino (seqüência 2) e consenso depositada no Pfam (seqüência 3). A região em amarelo foi selecionada para ser utilizada como isca. Para o alinhamento foi utilizada a ferramenta MATCH-BOX (http://www.fundp.ac.be/sciences/biologie/bms/matchbox_submit.html). O *score* sob o alinhamento (1 a 9) indica a confiabilidade, quanto menor o número mais confiável o alinhamento (Depiereux et al., 1997).

A seqüência do vetor pDBLeu contendo o domínio trombospondina murino (pDBLeu-thromb) utilizada como isca na triagem de biblioteca de duplo-híbrido foi confirmada por meio de amplificação por PCR (reação em cadeia da polimerase - *polymerase chain reaction*) das colônias de bactérias, seguida pelo seqüenciamento dos fragmentos com o tamanho correto. Para isso, foram utilizados os seguintes oligonucleotídeos: pDBLeu F (5'-GAATAAGTGCGACATCATCATC-3') e pDBLeu R (5'-GTAAATTTCTGGCAAGGTAGAC-3').

Uma das colônias apresentando a seqüência correta do inserto foi selecionada para a obtenção do DNA plasmidial por meio de Miniprep (Qiagen).

Transformação das Leveduras

Leveduras da linhagem MaV203 foram transformadas em pequena escala para a padronização da concentração de 3-amino-1,2,4-triazol (3AT) no meio de cultura e avaliação da eficiência de transformação. No total foram feitas cinco transformações (tabela 1). As transformações foram realizadas de acordo com o protocolo do sistema ProQuest ™ fornecido pela Invitrogen.

Tabela 1. Relação das transformações feitas em leveduras MaV203

Transformação	Vetor 1	Vetor 2	Meio seletivo
1	pDBLeu	-	SC-Leu
2	-	-	SC-Leu e SC-Leu-Trp
3	pDBLeu	pPC86	SC-Leu-Trp
4	pDBLeu-thromb	pPC86	SC-Leu-Trp
5	pDBLeu-thromb	-	SC-Leu

Padronização da Concentração de 3AT

Quatro colônias que cresceram em placas de ágar SC-Leu-Trp após cotransformação com os vetores pDBLeu-thromb e pPC86 (transformação 4) foram escolhidas para a verificação de ativação do gene repórter *HIS3* sem interação da isca com outra proteína e para a padronização da concentração de 3AT no meio de cultura.

Para isso, cada colônia foi ressuspendida em 100 µl de solução salina estéril (0,9% NaCl) e duas diluições seriais de 1:10 foram feitas a partir da suspensão de células inicial. Além das quatro colônias contendo os vetores pDBLeu-thromb e pPC86, foram selecionadas duas colônias de cada uma das linhagens controle A, B, C, D e E.

A determinação da concentração de 3AT necessária para inibir a expressão basal do gene repórter *HIS3* foi feita pingando-se 10 µl da suspensão inicial e das diluições de

cada colônia em placas contendo diferentes concentrações de 3AT na ausência de histidina. Foram utilizadas placas SC-Leu-Trp-His+3AT contendo 5 mM, 10 mM, 25 mM, 50 mM, 75 mM e 100 mM de 3AT. Para a determinação da quantidade de células em cada diluição, também foi feito o plaqueamento em meio SC-Leu-Trp, onde crescem leveduras que possuem os dois vetores independentemente da ativação de genes repórteres. As placas foram analisadas após incubação por 72 horas a 30°C.

A ativação direta ou indireta do gene *HIS3* pela isca é caracterizada pelo crescimento de colônias no meio de cultura contendo 100 mM de 3AT. Verificamos que não houve crescimento de colônias em uma concentração igual ou superior a 10 mM de 3AT no meio de cultura. Indicando que o domínio trombospondina não ativa a transcrição do gene *HIS3* sem que haja interação com uma proteína fundida ao domínio de ativação da proteína GAL4.

Foram realizadas duas triagens de biblioteca de cDNA, em cada uma foram utilizados 10 μg de DNA da biblioteca. Utilizamos o protocolo de transformação em larga escala fornecido pelo fabricante (Invitrogen) sem modificações. No total foram utilizadas 50 placas SC-Leu-Trp-His+3AT 10mM de 15 cm para avaliar a ativação do gene repórter *HIS3*, o que caracterizaria a interação do o domínio trombospondina com alguma proteína híbrida da biblioteca. Os plaqueamentos foram feitos utilizando-se esferas de vidro (Sigma). Para a determinação da eficiência de transformação, plaqueamos diluições seriais em meio SC-Leu-Trp, a contagem de colônias nessas placas permite a estimativa do número de células iniciais com os dois vetores.

Após a incubação das placas por sete dias a 30°C, as colônias obtidas foram replaqueadas em meio SC-Leu-Trp, as novas colônias foram utilizadas para se fazer um estoque em glicerol 25 % e para amplificação por PCR do vetor pPC86 utilizando-se oligonucleotídeos sugeridos pelo fabricante.

As amplificações foram realizadas incubando-se colônias individuais em 2 µL de tampão com liticase (50 mM Tris-HCl, pH 7.9; 10 mM MgCl2; 0.02 U/µL liticase) por 15 minutos a 30°C, seguindo então com a reação de PCR no mesmo tubo. Após a amplificação, seqüenciamos as amostras utilizando o *kit* DYEnamic ET Dye Terminator Cycle Sequencing e realizando a eletroforese em um MegaBACE 1000 (Amersham Pharmacia Biotech).

As seqüências obtidas foram analisadas com o auxílio da ferramenta BLAT (http://genome.ucsc.edu/), buscando regiões altamente similares no genoma do camundongo. Após a identificação do cDNA clonado, verificamos o quadro de leitura do inserto.

Resultados e Discussão

As transformações das leveduras com as bibliotecas permitiram-nos triar aproximadamente $2{,}3\times10^6$ clones de cDNA. A quantidade de transformantes recomendada para uma biblioteca de mamífero é de pelo menos 1×10^6.

Após a incubação por uma semana das placas, pudemos identificar 32 colônias que cresceram no meio seletivo para a interação, que provavelmente tinham o gene repórter *HIS3* ativo. O seqüeciamento do vetor pPC86 presente nessas colônias, no entanto, não revelou nenhuma possível interação, sendo todas as colônias falso-positivos (tabela 2).

O sistema de duplo-híbrido já foi utilizado anteriormente com sucesso para detectar interações de outras proteínas e domínios extracelulares: colágeno VI (Kuo *et al*., 1997); colágeno VII; laminina 5 (Aho *et al*., 1998); colágeno XVII (Aho *et al*., 1999); perlacan (Mongiat *et al*., 2002). Nestes trabalhos foi até identificada uma

interação com a trombospondina. Entretanto, nossa triagem não teve nenhum clone com uma possível proteína que interagisse com a isca.

Talvez a falta de interações identificadas seja devido a limitações do sistema de duplo-híbrido. Uma série de aspectos importantes para a interação entre proteínas não pode ser reproduzida por esse sistema, como modificações pós-traducionais ou o dobramento correto da proteína. Existe ainda a possibilidade do domínio trombospondina interagir com glicosaminoglicanas ou outras moléculas não peptídicas da matriz extracelular.

Tabela 2. Insertos identificados na triagem da biblioteca de duplo-híbrido

Número da colônia	Inserto do pPC86
1	*Zwint* (fora de fase)
2	*Prkar1b* (fora de fase)
3	*Serpini1* (3' UTR)
4	*Nme7* (fora de fase)
5	*Bc012020* (3' UTR)
6	Não foi possível seqüenciar
7	*Trim39* (3' UTR)
8	*Ociad1* (fora de fase)
9	*Eif2b2*
10	*Eif2b2*
11	*Col27a1* (fora de fase)
12	Sem RNAm identificado (sem ORF)
13	Não foi possível seqüenciar
14	Não foi possível seqüenciar
15	Seuqüência genômica (sem ORF)
16	Não foi possível seqüenciar
17	*Ak156652* (fora de fase)
18	*Ndufv1* (fora de fase)
19	*Lsm7* (fora de fase)
20	Não foi possível seqüenciar
21	*Rpli2* (fora de fase)
22	Não foi possível seqüenciar
23	*Crip2* (fora de fase)
24	*Gns* (3' UTR)
25	*Ash2l* (3' UTR)
26	Sem RNAm identificado (sem ORF)
27	Seuqüência genômica (sem ORF)
28	Não foi possível seqüenciar
29	*AK014152* (fora de fase)
30	Não foi possível seqüenciar
31	Não foi possível seqüenciar
32	Sem RNAm identificado (sem ORF)

Discussão Geral

A análise da região codificadora do gene *COL18A1* em 10 famílias não aparentadas possuindo pacientes portadores da SK levou à caracterização de oito alterações patogênicas nesse gene: c.12-2A>T, c.2969-2978delCAGGGCCCCC, c.3514-3515delCT, c.1238-1239insA, c.2105delC, c.3277C>T, c.2416C>T e c.3544+3A>C (Suzuki *et al*., 2002; Kliemann *et al*., 2003; Passos-Bueno *et al*., 2006; Keren *et al*., 2006). Essas alterações levam à criação de códigos de parada prematuros por diversos mecanismos mutacionais, tais como trocas de base que criam códigos de parada (*nonsense*), deleções e inserções que mudam o quadro de leitura do RNAm (*frameshift*) e mutações em sítios de excisão de introns (*splicing*). A identificação de novas mutações permitiu-nos confirmar que mutações no gene *COL18A1* são a causa da SK. Com a exceção da mutação de *splicing* c.12-2A>T, descrita anteriormente por Sertié *et al*. (2000), todas as outras alterações encontradas foram descritas pela primeira vez nestes trabalhos. Em três genealogias não foram encontradas mutações no *COL18A1* após a análise de todo o gene, sugerindo heterogeneidade genética para a síndrome. Entretanto, as mutações no gene podem não ter sido detectadas pela técnica empregada em função de algum outro mecanismo mutacional, como deleções de exons inteiros, rearranjos ou mutações em regiões promotoras.

As novas mutações identificadas, localizadas nos exons 10, 18, 23, 36, 40 e 41 devem causar degradação do RNAm, uma vez que mutações que criam códigos de parada prematuro não localizados no último exon provavelmente resultam na degradação do RNAm (Mendell and Dietz, 2001). Assim sendo, ao contrário do que ocorre na mutação de *splicing* do exon 2, que altera apenas uma das isoformas (Sertié *et al*., 2000), estas mutações devem levar à deficiência completa de todas as isoformas,

uma vez que estão presentes em exons compartilhados por todas as isoformas conhecidas do colágeno tipo XVIII.

Recentemente, pudemos comprovar a hipótese de que a SK é causada pela falta do colágeno tipo XVIII. A análise imunohistoquímica de biópsias de pele de dois irmãos portadores da mutação *nonsense* c.3277C>T em homozigose e de duas irmãs sem a mutação patogênica identificada, revelou uma deficiência quase completa da proteína (Capítulo 7; Suzuki *et al*., 2006).

Apesar do pequeno tamanho amostral de pacientes, parece haver uma correlação entre a gravidade do quadro clínico e a localização das mutações no gene *COL18A1*. Aparentemente os pacientes portadores da mutação c.12-2A>T possuem um menor comprometimento ocular, com perda da visão em uma idade mais avançada. Já os pacientes com mutações identificadas em outros pontos do gene tendem a perder a visão durante a infância e alguns deles apresentam problemas neurológicos. Nossa hipótese para explicar essa diferença no quadro clínico é a de que o comprometimento apenas da isoforma menor, esperado pela mutação c.12-2A>T, leva a um quadro mais leve, porém já é suficiente para resultar no fenótipo típico da SK. Nestes pacientes estão presentes as duas outras isoformas do colágeno XVIII, incluindo a maior que contém o domínio frizzled.

Recentemente, Duh *et al*. (2004) descreveram um paciente com 16 meses de idade e persistência de vasculatura fetal, a qual deve regredir para que a vascularização da retina ocorra normalmente. O paciente não possui níveis detectáveis de endostatina, sugerindo a perda completa do colágeno XVIII, e não apresenta nenhuma das mutações já descritas, no entanto a região codificadora do gene não foi analisada por completo. O defeito na regressão dos vasos hialóideos no desenvolvimento da vasculatura retiniana é uma característica marcante dos camundongos *col18a1*$^{-/-}$ (Fukai *et al*., 2002).

Interessantemente, Lobov *et al*. (2005) demonstraram a importância da sinalização WNT desencadeada por WNT7b mediada pelo receptor frizzled 4 na regressão de vasos hialóideos em camundongos. É possível, portanto, que o domínio frizzled do colágeno XVIII esteja de alguma forma envolvido na regressão da vasculatura fetal da retina e que sua falta resulte em um quadro ocular mais grave nos pacientes com SK.

O presente trabalho também foi o primeiro a descrever a existência do RNAm da isoforma mais longa (NC11-728) do colágeno tipo XVIII humano, bem como sua expressão em alguns tecidos, incluindo a retina. A isoforma NC11-728 apresenta em sua região N-terminal um domínio rico em cisteínas, homólogo às proteínas frizzled.

Como já mencionado anteriormente, o comprometimento de todas as isoformas parece levar a um quadro clínico mais grave, inclusive com a presença de alterações neurológicas. Kliemann *et al*. (2003) descreveram pela primeira vez alterações neurológicas em dois pacientes não aparentados com a SK, apresentando heterotopia subependimária nodular e dilatação ventricular. Recentemente, demonstramos que esses pacientes possuem uma deficiência quase completa do colágeno XVIII (Suzuki *et al*., 2006). Keren *et al*. (2006) descreveram uma paciente com agenesia de septo pelúcido, distúrbios de migração neuronal (paquigiria, polimicrogiria e heterotopia) e um leve retardo mental. A paciente possui a mutação c.3544+3A>C no sítio de *splicing* do exon 36, que acreditamos levar à ausência da proteína. Nosso trabalho, portanto, apresenta evidências de uma função das isoformas média e/ou longa do colágeno tipo XVIII na migração neuronal humana e no desenvolvimento do sistema nervoso central. Kuo *et al*. (2001) também observaram *in vitro* a capacidade do domínio NC1/endostatina de influenciar a migração de neurônios humanos. O envolvimento do colágeno XVIII com o desenvolvimento do sistema nervoso também já foi constatado em outros organismos. Ackley *et al*. (2001) demonstraram a influência dos domínos NC1 e endostatina na

migração neuronal e axonal em *Caenorhabditis elegans* e, mais recentemente, Schneider *et al*. (2006) apresentaram evidências da importância do colágeno XVIII para o crescimento de axônios motores em *Danio rerio*.

A variabilidade no tamanho da alteração occipital é observada intra e interfamilialmente e não parece estar associada com a falta de uma ou mais isoformas do colágeno tipo XVIII. Com a exceção da paciente portadora da mutação c.3544+3A>C, que apresenta um maior comprometimento neurológico, o fenótipo de todos os outros pacientes com mutações patogênicas no *COL18A1* não envolve comprometimento intelectual e todos apresentam defeito na região occipital e alterações oculares congênitas. Estes dados sugerem que o critério clínico mínimo para a suspeita de síndrome de Knobloch é a presença de alterações oculares congênitas associadas ao defeito na região occipital. Porém, dado o número restrito de pacientes testados e a dificuldade de triagem molecular dos pacientes que não cumprem estes critérios clínicos mínimos, é inviável um estabelecimento mais preciso do espectro da variabilidade clínica decorrente de mutações no *COL18A1*. A análise da proteína em biópsias de pele descrita neste trabalho mostra-se como uma possível estratégia para triagem de pacientes com um quadro clínico não característico da síndrome.

A triagem de mutações no *COL18A1* também resultou na identificação de cinco variações missense: T379M e L392P no domínio frizzled; I841V no domínio COL7; A1381T e D1437N no domínio endostatina (aqui indicados como A48T e D104N, de acordo com a numeração apenas da endostatina). Errera *et al*. (2006), estudaram as variantes polimórficas T379M e D104N em pacientes com Diabetes mellitus do tipo 2 e observaram uma associação entre a variante M379 e o desenvolvimento de obesidade nesses pacientes. Também foi constatada uma associação entre a variante M379 e a presença de retinopatia diabética proliferativa.

O domínio endostatina é a porção mais conservada do colágeno XVIII, tanto entre as proteínas ortólogas de outros organismos quanto o colágeno parálogo XV. Por esse motivo, alterações nessa região são boas candidatas a variantes funcionais. A variação polimórfica D104N foi associada ao desenvolvimento de câncer de próstata em 2001 por Iughetti *et al*. por meio de estudo de associação na população brasileira. Já a variante rara A48T da endostatina, foi identificada em homozigose em duas irmãs com o fenótipo característico da SK (Kliemann *et al*., 2003). Discutimos aqui a possibilidade dessas duas mudanças de aminoácido no domínio endostatina levarem a alterações funcionais.

Com base em nossos resultados de estudos funcionais com a mutação D104N, concluímos que essa troca de aminoácido não deve levar a uma alteração funcional da proteína. A ligação da endostatina com as moléculas testadas mostrou-se normal, ao contrário do resultado obtido por Menzel *et al*. (2004). Ainda é possível que esse polimorfismo resulte em mudanças na interação com proteínas não testadas, entretanto, existe uma série de evidências contra a hipótese de a variante N104 tratar-se de uma variante funcional (Liu *et al*. 2003; Ortega *et al*. 2003; Macpherson *et al*. 2004; Nascimento *et al*. 2004; Li *et al*. 2005; Stahl *et al*. 2005; Passos-Bueno *et al*. 2006). Menzel *et al*. (2004) sugeriram ainda um efeito patogênico para este polimorfismo, quando acompanhado de uma mutação nula no *COL18A1*. Entretanto, os nossos resultados não corroboram esta hipótese (Capítulo 4; Suzuki *et al*., 2005).

A endostatina T48, no entanto, parece ter uma função alterada. A afinidade a algumas das moléculas testadas é significativamente inferior quando comparada à da endostatina normal, apesar da modelagem molecular não indicar uma alteração na proteína. As diferenças constatadas não são suficientes para concluir se a SK pode ser causada por esta alteração, por ser uma doença causada pela deficiência total do

colágeno XVIII. Em 2005, Stahl *et al*. produziram a endostatina T48 humana em células de mamífero e observaram uma redução de 40 % na produção da proteína mutada, sugerindo uma redução na eficiência de dobramento. Não se sabe se o problema de dobramento constatado é capaz de prejudicar o colágeno XVIII a ponto dos níveis da proteína serem tão reduzidos quanto dos pacientes com mutações nulas detectadas (Capítulo 7; Suzuki *et al*., 2006)

A função do domínio trombospondina, localizado na porção N-terminal do colágeno XVIII é totalmente desconhecida. Com o intuito de contribuirmos para a elucidação funcional desta região, propusemo-nos a identificar proteínas que pudessem interagir com essa região do colágeno utilizando a técnica de duplo-híbrido em leveduras. Em duas triagens de bibliotecas de cDNA (embrião de camundongo total, E10.5), foram isoladas 32 colônias com crescimento em meio seletivo, entretanto, após o seqüenciamento, todas as colônias foram identificadas como sendo falso-positivos. Esse insucesso na identificação de proteínas que interagem com o domínio trombospondina deve-se provavelmente às limitações do sistema de duplo-híbrido. Uma série de aspectos importantes para a interação entre proteínas não pode ser reproduzida por esse sistema, como algumas modificações pós-traducionais ou o dobramento correto da cadeia polipeptídica. Existe ainda a possibilidade do domínio trombospondina interagir com glicosaminoglicanas ou outras moléculas da matriz extracelular.

Em conclusão, nosso trabalho confirmou a falta do colágeno tipo XVIII como a principal causa da SK, sugerindo ainda a existência de heterogeneidade genética para a doença. A caracterização de novas mutações no *COL18A1* permitiu-nos incluir as alterações neurológicas nos possíveis sinais clínicos da SK e dessa forma, apresentar evidências do envolvimento do colágeno XVIII na migração neuronal humana. A medição dos níveis de endostatina plasmática por ELISA não se mostrou uma técnica

muito segura, enquanto a imunohistoquímica mostrou uma acentuada redução na marcação do colágeno XVIII nos pacientes. Assim sendo, sugerimos a utilização de marcação imunohistoquímica do colágeno XVIII em biópsias de pele como um possível teste diagnóstico, Finalmente, demonstramos que a variante N104 da endostatina provavelmente não apresenta alterações funcionais, enquanto a variante T48 da endostatina mostrou-se alterada em sua interação com outros componentes da matriz extracelular, sendo possivelmente uma variante funcional da proteína. Com isso, concluímos que são necessários estudos funcionais da proteína além da modelagem molecular *in silico*.

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