AN INVESTIGATION INTO THE MOLECULAR BASIS OF FAMILIAL FORMS OF OSTEOARTHROPATHY IN SOUTH AFRICA

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A thesis submitted to the University of Cape Town in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Human Genetics, Faculty of Medicine.

August, 1998

THIS THESIS IS DEDICATED TO MY FAMILY, WHOSE CONFIDENCE AND CO-OPERATION HAS ENCOURAGED ME.

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ABSTRACT

August, 1998

An investigation into the molecular basis of familial forms of osteoarthropathy in South Africa

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Generalised osteoarthritis (OA) is a common disorder of the joints which can lead to pain and disability. Identification of the determinant gene(s) is limited in part by the lack of Mendelian inheritance in most forms of the disorder, the combination of genetic and environmental influences and the late development of the condition. An approach to the investigation of the aetiology of OA would be to take advantage of the monogenic basis of inherited skeletal dysplasias in which OA is a major component. For this reason, the molecular genetic basis of the epiphyseal dysplasias, which encompass a spectrum of phenotypes ranging from mild to severe skeletal involvement, is addressed in this thesis. Familial skeletal disorders in South Africa in which OA is a major feature were identified and investigated using intragenic and closely linked microsatellite markers in order to determine linkage to candidate genes. Mutational analysis was undertaken to identify the genetic defect.

The candidate genes were selected on the basis of the involvement of their products in the structural integrity of articular cartilage, the primary tissue which is affected in the osteoarthropathies. The most likely candidate for the epiphyseal dysplasias was the COL2A1 gene, which encodes type II collagen, the major component of articular cartilage. Other candidate genes in the present investigation included those encoding the minor collagenous components, namely, types IX and XI collagen, the genes encoding the collagen of the growth plate, type X collagen and those encoding the

the collagen of the growth plate, type X collagen and those encoding the type VI collagen monomers. Screening of the genes encoding the proteoglycans aggrecan, decorin, fibromodulin and lumican, and the genes encoding other non-collagenous proteins of the extracellular matrix such as cartilage oligomeric matrix protein, cartilage link protein, cartilage matrix protein, chondrocalcin and matrix metalloproteinase 3, was also undertaken.

In some of the rare epiphyseal dysplasias, phenotypic features include high myopia and deafness, as in the Stickler syndrome type 1. In the present study, a COL2A1 haplotype was found to cosegregate with a condition resembling Stickler syndrome type 1 in an Afrikaner family which also manifested atypical brachydactyly. The underlying molecular defect proved to be a C2503T transition which resulted in an Arg704Cys substitution. The mildness of the condition in this family, compared to the greater skeletal involvement in other SED phenotypes which are caused by Arg-Cys substitutions, could possibly be attributed to the variant position of the change within the Gly-X-Y repeat of the triple helix.

The COL2A1 gene was also implicated in two large kindreds from the western Cape province who exhibited a form of spondyloepiphyseal dysplasia (SED). These respective disorders, SED type Cape Town (SEDCT) and Namaqualand SED (NSED), both presented with dysplasia of the femoral capital epiphyses and severe secondary degenerative OA which developed in early adulthood. The clinical and radiographic phenotypes in both families were similar, differing only in the respective stature of the affected individuals. The disorder in each kindred was found to cosegregate with the same COL2A1 haplotype. Mutation analysis revealed a Gly472Cys substitution in the triple helix of the type II collagen protein in affected persons from both families. The common mutation implies that these two conditions are the same entity and that a founder effect is operative.

In another family with the mild, Ribbing type of multiple epiphyseal dysplasia (MED), no recombinants were found using markers close to the COMP gene.

Mutation screening revealed a C1594G transversion in exon 14 which encodes a calmodulin-like repeat of the COMP molecule. The mutation resulted in an Asn523Lys substitution which is thought to alter the calcium binding capacity of the COMP molecule.

Familial primary generalised OA (FOA) in a large family was investigated for linkage to eighteen possible candidate genes. Of those, twelve were excluded on the basis of LOD scores <-2.00 at theta=0.00 and meiotic recombination, from being involved in the aetiology of the condition. The involvement of the other six genes could not be determined conclusively. The findings of this investigation indicate that a potential candidate for FOA may exist at a locus within 5cM of the COL11A1 gene.

Mseleni joint disease (MJD) is a severe form of OA which has an unusually high incidence in a geographically isolated population. An association to candidate genes expressed in cartilage, was investigated by comparing allele frequencies in affected and unaffected groups of people from the Mseleni district. Although there was no obvious linkage disequilibrium with the candidate genes, an increased homozygosity for the alleles of an intragenic COL9A1 marker was observed and warrants further investigation. Histological analysis showed an accumulation of type VI collagen around the chondrocytes.

Although no clear conclusions can be drawn concerning the overall contribution of genetics to the common form of OA in the population, the findings presented in this thesis will contribute towards the understanding of the different genes which are involved in OA and the mechanisms by which the condition can arise. These molecular findings also provide a means of management in the affected individuals by virtue of presymptomatic diagnosis and planning suitable lifestyles for individuals who are at risk of manifesting these skeletal dysplasias.

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ABBREVIATIONS AND SYMBOLS

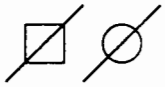
AD	autosomal dominant
AGC1	gene encoding aggrecan
Ala	alanine
AR	autosomal recessive
Arg	arginine
Asn	asparagine
ASP	affected sibpair analysis
Asp	aspartic acid
autorad	autoradiograph
bp	base pair/s
°C	degree Celsius
C	collagenous
Ch.	chapter
C-propeptide	carboxyl terminal propeptide
C2503T	substitution of cytosine at position 2503 by thymine
Ca^{++}	calcium ions
[CA]n	CA dinucleotide
CCAL2	chondrocalcin gene
CRTM	cartilage matrix protein
CRTL1	cartilage link protein
cM	centiMorgan
COMP	cartilage oligomeric matrix protein
COOH-term	carboxyl terminus
CSGE	conformation sensitive gel electrophoresis
DCN	decorin
dCTP	α-deoxycytosine-triphosphate

DNA	deoxyribonucleic acid
EDM 1	MED locus 1 on chromosome 19
EDM2	MED locus 2 on chromosome 1
EGF-like	ectodermal growth factor-like
et al	and others
FACIT	fibril-associated collagens with interrupted triple helices
FMOD	fibromodulin
FOA	familial generalised osteoarthropathy
Glu	glutamate
Gly	glycine
HJD	Handigodou disease
hrs	hours
kb	kilobase/s
kD	kilodaltons
KcM	Kosambi centiMorgans
lod	log of the odds
LUM	lumican
Lys	lysine
M	molar
mM	millimolar
µg	microgram
MDE	gel matrix
MED	multiple epiphyseal dysplasia
min	minutes
ml	millilitre(s)
µl	microlitre(s)
MJD	Mseleni Joint disease
MMP3	matrix metalloproteinase 3
N-propeptide	amino terminal propeptide

NC	non-collagenous
NH_2. term	amino terminus
NSED	Namaqualand spondylo-epiphyseal dysplasia
OA	osteoarthropathy
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PSACH	pseudoachondroplasia
RER	rough endoplasmic reticulum
RFLP	restriction fragment length polymorphism
SED	spondylo-epiphyseal dysplasia
SEDC	spondylo-epiphyseal dysplasia congenita
SEDCT	spondylo-epiphyseal dysplasia type Cape Town
Ser	serine
SSCP	single strand conformational polymorphism
STK	Stickler syndrome type 1
UCT	University of Cape Town
UK	United Kingdom
UTR	untranslated region
Val	valine
VNTR	variable number of tandem repeats
W	watts

affected/ unaffected male

affected/unaffected female



deceased male/female

SECTION I

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: LITERATURE REVIEW

CHAPTER 1:
GENERAL INTRODUCTION

"...our knowledge of the disease is incomplete, perhaps because it is one of those dull commonplace disorders that are hard to study with enthusiasm, but new knowledge of osteoarthritis must be gained if the later years of our lengthening lives are not to be plagued by increasing pain and disability".

JH Kellgren, 1961

Osteoarthritis (OA) is the end result of a number of pathophysiological processes, hereditary or environmental, which all have a common pathway of cartilage destruction. It is a common disorder of joints which, in many instances, causes progressive loss of motion. Despite considerable progress in the understanding of OA over the past three decades, there is still a pressing need to unravel the aetiology of the condition, in order to identify risk factors for the development and progression of OA, and to devise primary prevention strategies.

The available evidence suggests that genetic factors have a major role in OA. The nature of the genetic influence is, however, speculative and may involve a structural defect, alterations in cartilage or a genetic influence on a known risk factor. The role of genetic factors in the development of OA has attracted extensive research which has been facilitated by the advances in molecular biology.

Because of the difficulty in separating out the individual factors which contribute towards the phenotype of a multicomponent condition such as OA, researchers have often opted to study monogenic disorders in which OA is a major feature, but which show clear mendelian inheritance. The advantage of investigating these latter conditions is that they result from a single gene defect and thus provide the best approach to isolating the different mechanisms which can contribute toward OA. The general opinion of researchers in this field is that genetic studies of well-characterised families expressing common forms of OA, may give important, possibly novel, insights into pathogenesis. In this regard, the present South African study involved the investigation of kindred with rare skeletal dysplasias in which OA is a component.

1.1. PLAN OF THE THESIS

This thesis is divided into eight chapters. The first chapter provides an introductory background to the osteoarthropathies and provides a general overview of the aim of the study and the scientific approach to the investigation.

The second chapter introduces articular cartilage which is assumed to be defective in the osteoarthropathies. The role of the various candidate genes in the aetiology of skeletal abnormalities is discussed, thus providing scientific reason for nominating them as candidates for the genetic conditions covered by this thesis. In Chapters 3-7 each of the unique osteoarthropathies encountered in the South African population is discussed separately, providing background information on the clinical and

radiological findings and discussing the molecular investigations undertaken in this project. The thesis is concluded in the eighth chapter, where the findings of the molecular studies in the South African families are summarised and the scientific contribution which these investigations have made towards our understanding of OA, is discussed. Future prospects for further investigations are addressed.

1.2. INTRODUCTORY BACKGROUND TO OSTEOARTHROPATHY (OA)

Generalised OA is a degenerative disorder which affects multiple bones and joints, the most commonly affected being the large weight-bearing joints such as the hips and knees together with the hands, spine and feet. The condition is characterised by destructive lesions in articular cartilage which progresses to a loss of this tissue and may eventually lead to exposure of the subchondral bone (Fig.1.1).

Osteoarthropathy is not a single entity but could be regarded as the final common pathway of cartilage degradation [Freeman, 1980]. The aetiology of the common, ostensibly non-genetic form of OA is unknown but epidemiologic studies have revealed a relationship between age and other contributing factors such as occupation and lifestyle [Mankin et al., 1986; Hochberg, 1991; Felson, 1993; Tikly, 1994]. The issue surrounding the heritability of generalised OA is controversial and has sparked much debate. For a long time, it was considered that familial OA was secondary to the effects of skeletal abnormalities or chondrodysplasias. Evidence has

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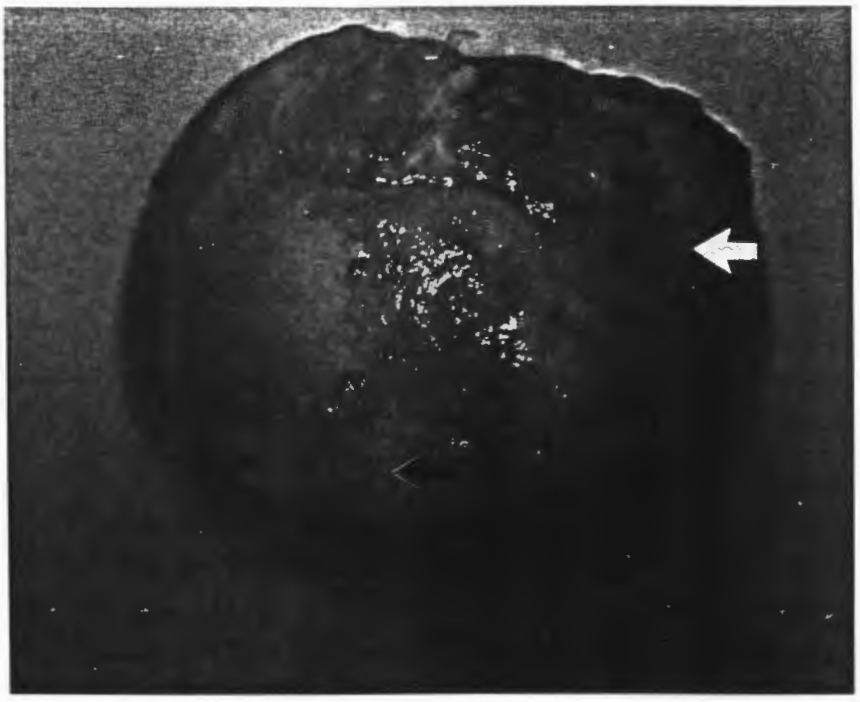

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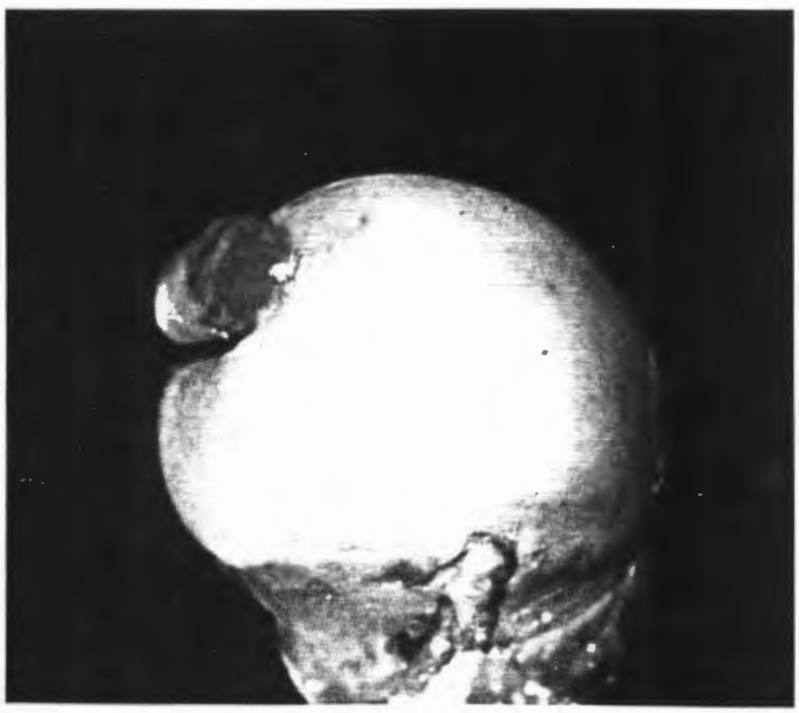

Fig.1.1. Photographs comparing the morphology of the articular cartilage surface of femoral heads from hip specimens (a) with and (b) without osteoarthropathy. The erosion of the articular cartilage down to the subchondral bone is depicted in (a) (white arrow- original remaining cartilage; black arrow - eroded region). The hip specimens were obtained at the time of hip replacement surgery and postmortem, respectively.

accumulated to suggest, however, that an inherited predisposition may be an important factor in the development and progression of some forms of generalised OA [Stecher et al., 1953; Kellgren et al., 1963; Prockop et al., 1997]. Much knowledge has been gained concerning the structural collagenous and non-collagenous components of articular cartilage and their disruption in some disorders. This thesis was undertaken to investigate, at a molecular level, the aetiology of familial chondrodysplasias of which OA is a major feature, which had been encountered in the South African population.

1.3. THE PROBLEM TO BE INVESTIGATED: FAMILIAL OA IN SOUTH AFRICA

Conventional "non-genetic" generalised OA is as prevalent in the elderly South African population as it is worldwide, affecting 15-20% of people over the age of 50 years [Reynolds et al., 1992]. In addition, several large southern African kindreds suffer from early onset familial degenerative arthropathy of the hip [du Toit, 1979; Beighton et al., 1984; Ramesar and Beighton., 1992].

The different inherited osteoarthropathies vary from each other by virtue of their natural history and the anatomical distribution of the joints which are affected. All except one of the conditions chosen for this study follow an autosomal dominant pattern of inheritance and are therefore perpetuated from generation to generation, affecting large numbers of individuals within the kindreds. The following conditions in this category have been investigated: Namaqualand Hip dysplasia (NSED),

spondyloepiphyseal dysplasia type Cape Town (SEDCT), multiple epiphyseal dysplasia (MED) and a syndrome resembling arthro-ophthalmopathy (Stickler syndrome type I). A familial form of autosomal dominant osteoarthropathy (termed FOA for the purposes of this study) with no other syndromic features and affecting several individuals in a large family, has also been investigated. Another disorder which has been addressed in this study, is Mseleni joint disease (termed MJD for the purposes of this study). This disorder is a severe, chronic form of OA encountered in southern Africa, which affects several hundred people within a small remote region of the province of Kwa-Zulu Natal in South Africa. Although MJD clusters in families, it does not have a clear Mendelian pattern of inheritance.

1.4. THE AIM OF THIS STUDY

The aim of this study was to elucidate the molecular genetic basis of disease in familial forms of OA in the South African population. In order to achieve this objective, investigations were undertaken to determine whether the OA phenotypes were associated with candidate genes which code for the major components of articular cartilage. Thereafter the genes in question were screened for disease-predisposing mutations. The goal of this study was therefore to identify the defective gene and characterise the pathogenic mutation in each of the osteoarthropathies to be investigated.

1.5. THE APPROACH TO THIS STUDY

Inherited forms of skeletal abnormalities have been a focal point of interest in the Department of Human Genetics, UCT, for more than 25 years. Numerous families and sporadic individuals with skeletal abnormalities had been documented at clinics during that period. Affected individuals were thoroughly examined, both clinically and radiologically. In order to determine the extent of the skeletal involvement and to make an accurate diagnosis, blood specimens were obtained from the available family members and their DNA was banked to allow for subsequent molecular investigations.

In order to pursue molecular analyses, it was necessary, in some instances, to obtain blood specimens from additional family members. Molecular investigations included linkage analysis to determine whether the OA stigmata were cosegregating with any of the candidate genes. This approach involved the use of polymorphic intragenic markers for accurate colocalization of the disorder and candidate gene and in order to reduce the risk of recombinations. Microsatellite DNA markers from the vicinity of the chromosomal regions of the candidate genes were used if the intragenic marker was not informative or not available. Two-point LOD scores were calculated using a computer programme to determine whether there was an association between the condition and the DNA marker. In those instances where no recombinant events were observed between the phenotypic status and DNA markers in family members, the candidate gene in that chromosomal region was screened for exonic mutations by single stranded conformational polymorphism (SSCP) analysis. Variations from the

expected SSCP mobility pattern, suggesting a possible mutation, were verified by direct sequencing of the exons. These variants were compared with the known nucleic acid sequences and the effect of the sequence change upon the protein product was deduced.

CHAPTER 2:

LITERATURE REVIEW; THE BIOLOGY OF ARTICULAR CARTILAGE

2.1. GENERAL BACKGROUND

Cartilage is a highly specialised avascular structure which is widely distributed in the human body and which plays an important role in the formation and growth of the skeleton. The cells that produce the cartilage matrix are the chondroblasts or chondrocytes. The development of the human skeleton involves two stages: firstly, the formation of a cartilage model - a process of cell differentiation and pattern formation; secondly, the replacement of this cartilage by bone during endochondral ossification - the latter process involves cartilage maturation (hypertrophy), matrix degradation, vascular invasion and ossification [Erlebacher et al., 1995].

This study is focussed on articular cartilage, which is the thin layer covering the bone ends which articulate in joints. The chain of events observed in the development of OA is best explained by the assumption that the primary abnormality is in the articular cartilage and that all pathological changes in the other joint structures are attributable, directly or indirectly, to these cartilage changes.

The mechanical properties of cartilage and its ability to withstand pressure and allow low friction movement, are determined by its components, which are mainly collagen and proteoglycans. During cartilage synthesis the chondrocytes secrete and assemble a highly complex extracellular matrix in which the fibrillar collagen framework and proteoglycans interact. A dense macromolecular network is created which imparts structural integrity and resilience to the cartilage tissue. The most abundant macromolecules are type II collagen which comprises about 85% of the total collagen content, together with aggregates of aggrecan and hyaluronic acid [Byers, 1993]. Several other collagens, small proteoglycans and non-collagenous components are covalently bound to each other and contribute towards the stability of the cartilage matrix.

2.2. COLLAGENOUS COMPONENTS OF ARTICULAR CARTILAGE

The collagens are a complex family of secreted molecules that share similar triple-helical domains and are comprised of closely related proteins which are genetically distinct [Eyre et al., 1987a]. Cartilage displays an extensive variety of collagen types, the most widespread and abundant being the fibrillar collagens viz. types I, II, III, V and XI. This thesis will focus on molecular genetic investigations of the integrity of the genes encoding types II and XI collagen, as well as those encoding the fibril-associated type IX collagen, the network collagen, type X, and type VI collagen of microfibrils [Byers, 1993].

The distinctive feature of every collagen molecule is its unique protein structure which comprises highly repetitive sequences of amino acids in the format: glycine (Gly)-X-Y [Prockop, 1992]. The length of the uninterrupted repeating tripeptide unit varies among collagens, ranging from 338 to 341 triplets in the fibril-forming proteins, and being considerably shorter in the other collagen subgroups. The amino acid in the X position is frequently proline and the Y position is frequently occupied by hydroxyproline. The presence of a glycine residue at every third position enables the collagen molecule to form a helical structure. The glycine is in the center of the triple helix, occupying a restricted space which can only accommodate this small amino acid. Glycine does not contain a bulky side group and the chains are able to interact with each other at the glycine residues. If the side-chain of any substituting amino acid disrupts the triple helix, the consequences are highly deleterious [Williams and Jimenez, 1995a; Horton, 1996; Rimoin, 1996] (Table 2-I).

In order to achieve the tightest fit, the 3 polypeptide chains of the collagen presursor fold and twist into the triple helical conformation which is crucial for transport of the fibrillar collagens beyond the rough endoplasmic reticulum (RER). The triple helix is stabilised by interchain hydrogen bonds between the amide group of glycine and the oxygen of the carbonyl group of an amino acid in the X position in an adjacent chain. Further stabilisation occurs as a result of hydrogen bonds between the hydroxyl group of hydroxyproline and the carbonyl backbone of the chain [Mathews and van Holde, 1990].

Each collagen is made up of 3 polypeptide α chains wound into a characteristic left-handed triple helix. The molecule may be composed of only one type of α chain (i.e. a homotrimer as in type II collagen) or 2 or all 3 chains may be different (collagen types VI, IX, XI) [Horton, 1996]. By convention, each of the collagens are identified by roman numerals and the individual α chains of the triple helices are represented by arabic numerals. Categorisation of the collagens is based on their α chain characteristics, molecular assembly and supramolecular structures [Murray, 1986].

2.2.1. Type II collagen

This is the major collagenous component of all hyaline cartilage, nucleus pulposus and vitreous humour [Ayad et al., 1994]. It forms the network of fibrils upon which the other matrix components can be deposited [Mayne, 1989]. Type II collagen is a homotrimer containing three identical α1(II) chains which form a continuous triple helix. Chondrocytes are the main cells expressing the single gene, COL2A1, which encodes all 3 chains of the helical molecule. COL2A1 has been localised to 12q13.11-q13.12 [Takahashi et al., 1990] between the microsatellite markers D12S331 and D12S339 (The Human Gene Map http:/ /www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96; Fig. 2-1). The combined efforts of a number of investigators has led to the elucidation of the structure of the COL2A1 gene [Strom and Upholt, 1984; Cheah et al., 1985; Sangiorgio et al., 1985; Vikkula and Peltonen et al., 1989; Ala-Kokko and Prockop, 1990].

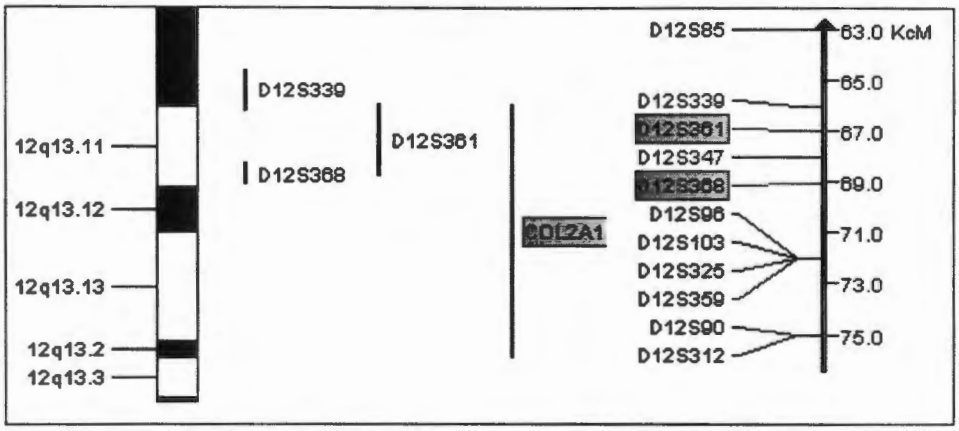

Fig. 2-1. Representation of 12q13.11-q13.12 and the approximate location of the microsatellite markers (within 5cM of the candidate region for the COL2A1 gene) which were used to show linkage to COL2A1. The genetic distances between the markers (shaded) and relative to other markers, are shown in Kosambi centimorgans (KcM). This figure incorporates data taken from GDB (http://www.gdb.org).

The gene is about 30kb in size and transcribes into a 6kb mRNA transcript. The 54 exons encode three functional protein domains: exons 1 to 6 encode the N-terminal propeptide, exons 6-49 encode the triple helical domain, and exons 49 to 52 encode the C-terminal propeptide. All exons encoding the main triple helix contain 54bp or multiples thereof. Furthermore, the exons contain complete codons and encode complete Gly-X-Y triplets [Cole, 1994]. An intragenic VNTR in the 3' untranslated region of the gene [Stoker et al., 1985] and two closely linked markers, D12S361 and D12S368, were used for linkage in the present study.

Mutations in the COL2A1 gene have been implicated in skeletal dysplasias which involve the tissues where the type II collagen gene is expressed. The type II collagenopathies now include the entire family of spondylo-epiphyseal dysplasias (SEDs) which involves the spine and epiphyses. These conditions comprise an extensive spectrum of phenotypes ranging from the milder SED forms and the

Stickler syndrome to the severe hypochondrogenesis and achondrogenesis phenotypes [Cole, 1994; Horton, 1996; Rimoin, 1996].

The most common type of mutation which occurs in the COL2A1 gene appears to involve glycine substitutions in the collagen triple helix. Early biochemical studies of cartilage from persons with various forms of chondrodysplasia provided evidence for a gradient of phenotypic severity which relates to changes in the triple helix; generally, mutations occuring close to the carboxyl terminus result in the more severe chondrodystrophies whereas in milder phenotypes, mutations occurred closer to the amino terminus. This gradient is demonstrated in individuals with Gly-Ser substitutions which produce phenotypes ranging from severe achondrogenesis type II (Gly691,943Ser) to mild forms of SED with precocious osteoarthritis (Gly247,274,443Ser) [Horton et al., 1996]. Some mutations which cause a SED phenotype such as Gly895, 997Ser, do not, however, fit the gradient pattern [Winterpacht et al., 1995; Chan et al 1991].

The mutations which have been described in the COL2A1 gene include insertions, deletions and base substitutions. Amongst these are the termination mutations which result in stop codons within the triple helix. In this instance, the resultant truncated collagen monomer cannot be incorporated into the collagen fibre and therefore does not have the same deleterious effect of mutant strands which become part of the mature collagen molecule. Instead, the loss of collagen monomers, or haploinsufficiency, results in Stickler syndrome type 1, which is a mild skeletal

dysplasia and is characterised by severe myopia and hearing loss [Zlotogora et al., 1992]. The COL2A1 gene also appears to have mutation hotspots which are prone to Arg-Cys substitutions, in particular Arg 75 and Arg 519, that result in a mild form of SED with precocious OA [Williams et al., 1995b; Bleasel et al., 1995]. Other type II collagenopathies include mild forms of OA (early and late onset), severe forms of chondrodysplasia such as SED congenita and Kniest dysplasia and the lethal achondrogenesis type II and hypochondrogenesis phenotypes [Beighton, 1988].

2.2.2. Type VI collagen

Type VI collagen is a short-helical heterotrimer which is widely expressed in connective tissue. The molecule is composed of 3 genetically distinct α chains encoded by 3 different genes viz. COL6A1, COL6A2 and COL6A3. The COL6A1 and COL6A2 genes form a gene cluster within 185kb on 21q22.3 [Weil et al., 1988] (Fig. 2-2a) and may be separated by as much as 150kb [Heiskanen et al., 1995]. The two genes occur as a gene pair in head-to-tail configuration. The COL6A1 gene is 29kb long (at this time, the exact number of exons is not known) and the COL6A2 gene is 36kb in length, comprising 30 exons. An intragenic marker and the microsatellite markers , D21S171 and D21S1446, were selected for analysis in the present study. The gene encoding COL6A3 has been localised to a 17cM region on 2q37 between the markers, D2S336 and D2S395 [The Human Gene Map]. Markers D2S338 and D2S345, within this region, were used in the present study (Fig. 2-2b, Table 2-I).

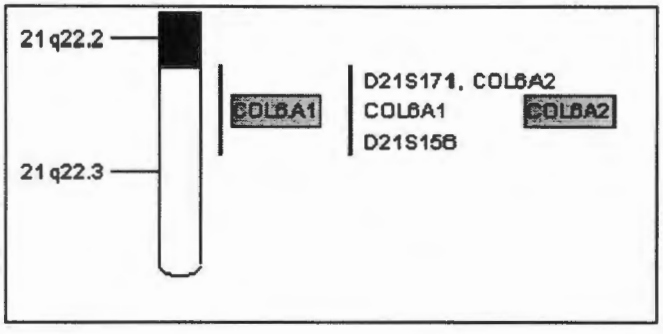

Fig. 2-2a. Representation of 21q22.2-q22.3 and the approximate location of the intragenic and microsatellite markers (within 5cM of the candidate region for the COL6A1 and COL6A2 genes) which were used to show linkage to COL6A1 and COL6A2. This figure incorporates data taken from GDB (http://www.gdb.org).

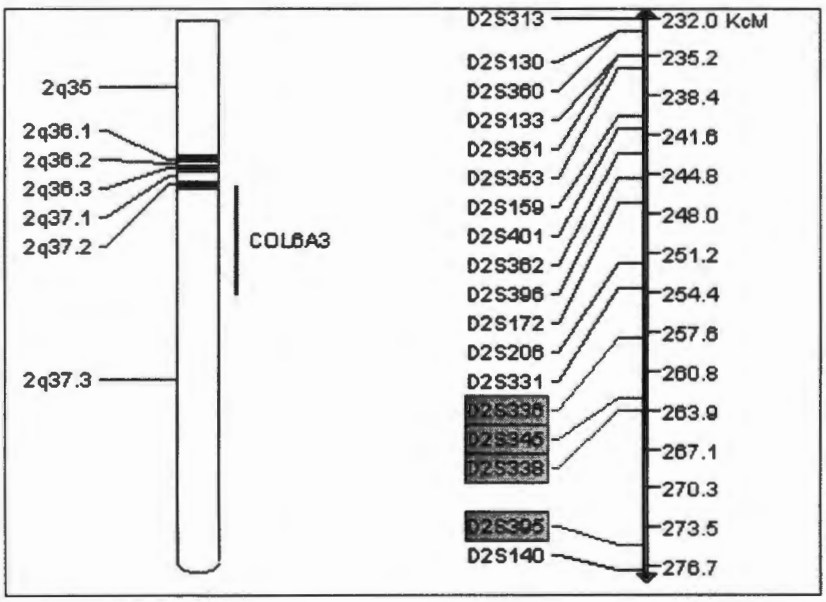

Fig. 2-2b. Representation of 2q36-q37 and the approximate location of the microsatellite markers (within 5cM of the candidate region for the COL6A3 gene) which were used to show linkage to COL6A3. The genetic distances between the markers (shaded) and relative to other markers, are shown in Kosambi centimorgans (KcM). This figure incorporates data taken from GDB (http://www.gdb.org).

Several observations have been made which imply that type VI collagen has a role in cell attachment and associates with the major collagen fibrils [Keene et al., 1988; Jobsis et al., 1996]. It has also been suggested that the type VI collagen triple helix is specifically adjusted to form tissue-bound microfibrils which are considered to have an anchoring function [Chu et al., 1988].

Base substitutions in the α1(VI) and α2(VI) collagen chains have been associated with Bethlem myopathy, an autosomal dominant disorder with contractures which affects skeletal muscle [Jobsis et al., 1996]. In 4 families the mutations involve Gly-Ser/Val changes which disrupt the Gly-X-Y motif of the collagen triple helix. The COL6A3 gene product has also been implicated in Bethlem myopathy by genetic linkage analysis in a large family [Speer et al., 1996].

2.2.3. Type IX collagen

Type IX collagen belongs to the FACIT (fibril-associated collagens with interrupted triple helices) subfamily of collagens and contains collagenous (C) and non-collagenous (NC) domains. The molecule can also be regarded as a proteoglycan as some of its forms have glycosaminoglycans attached to their non-collagenous domains [McCormick et al., 1987]. Type IX collagen is co-distributed throughout the extracellular matrix with type II collagen fibrils and may be concentrated where the fibrils intersect [Muller-Glauser et al.,1986]. Furthermore, type IX collagen may co-polymerise with type II fibrils, and possibly mediates physical interaction between collagen fibrils and proteoglycans in cartilage [Eyre and Wu, 1995]. It is possible that the incorporation of type IX collagen onto the surface of fibrils might serve to control

lateral fibril growth and the loss or absence of type IX collagen may result in fibril aggregation. Smith and Brandt [1992] proposed that type IX may be an intermediary molecule which is necessary for network stabilization.

The type IX collagen molecule consists of a heterotrimer, with 3 distinct gene products which assemble to form a single molecule of chain composition α1(IX)α2(IX)α3(IX). The gene encoding the α1 chain, COL9A1, contains 19 exons and spans 100kb on the chromosomal region 6q12-q13 [Kimura et al, 1989; Warman et al., 1993a]. In the present study, intragenic microsatellite markers, 509-12B1 and 509-8B2, were used to demonstrate linkage to the gene. The COL9A2 gene has been localised to 1p32.3-p33 [Warman et al., 1994; Hellsten et al., 1995] between the markers D1S255 and D1S2861[The Human Gene Map], and has 32 exons spanning about 10kb [Muragaki et al., 1990]. The most informative marker, situated in the closely linked MYCL1 gene (Fig. 2-3a), was used to show linkage in the present study. More recently, the gene encoding COL9A3 was localised to 20q13.3 [Brewton et al., 1995]; the structure of the gene is unknown. Closely linked markers, D20S171 and D20S64 [Research Genetics] (Fig. 2-3b) were used for linkage analysis in the present study.

The COL9A1 gene has been implicated in chondrodysplasias as demonstrated in mice with non-inflammatory degenerative joint disease [Fassler et al., 1994]. Further confirmation of the importance of the α1(IX) chain for normal cartilage function was obtained by Hagg et al. [1997] who reported a functional knock-out of the entire type IX collagen protein in the absence of the α1(IX) chain.

Initial studies by Briggs et al. [1994] demonstrated an association between COL9A2 and the severe, Fairbank form, of multiple epiphyseal dysplasia (MED) which is characterised by generalised abnormalities of the epiphyses, stunted stature and brachydactyly [Fairbank, 1946]. By identifying a heterozygous mutation in a Dutch family with MED, Muragaki et al. [1996] confirmed that COL9A2 was a locus for MED. The mutation involved a splice donor site which resulted in exon skipping within the triple helical domain of the gene.

As yet, no chondrodystrophy has been found to be associated with abnormalities in COL9A3.

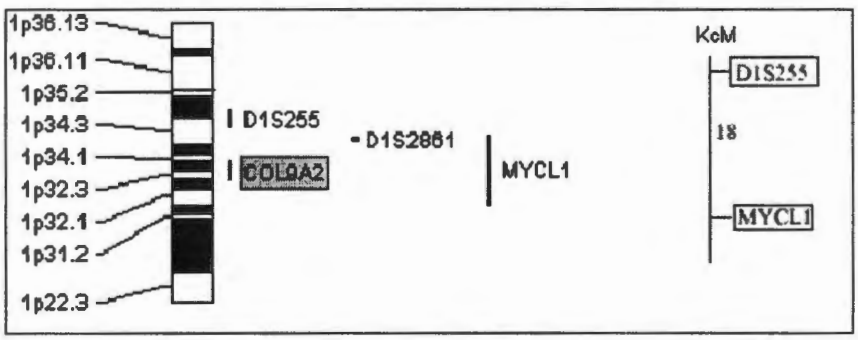

Fig. 2-3a. Representation of 1p32.3-p33 and the approximate location of the microsatellite markers (within 5cM of the candidate region for the COL9A2 gene) which were used to show linkage to COL9A2. The genetic distances between the markers (shaded) and relative to other markers, shown in Kosambi centimorgans (KcM), were obtained from the CEPH/Généthon linkage map. This figure incorporates data taken from the GDB (http://www.gdb.org).

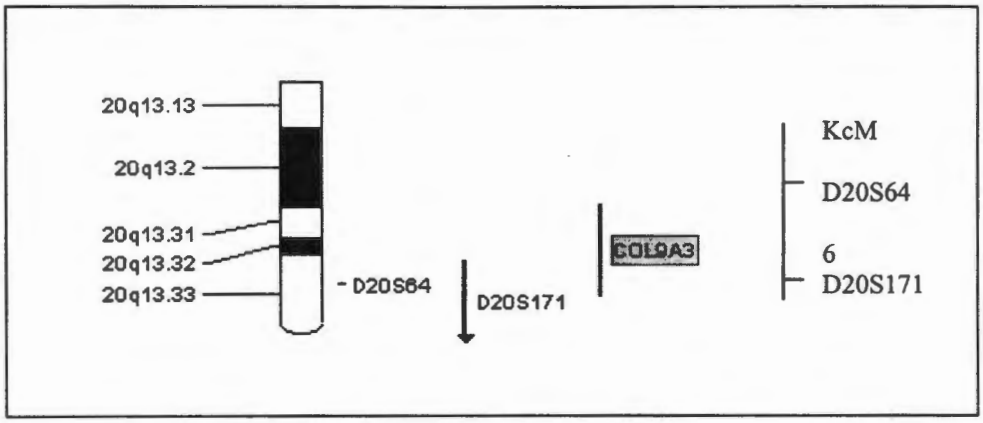

Fig. 2-3b. Representation of 20q13.2-q13.33 and the approximate location of the microsatellite markers (within 10cM of the candidate region for the COL9A3 gene) which were used to show linkage to COL9A3. Genetic distances in Kosambi centimorgans (KcM) were obtained from the CEPH/Généthon linkage map. This figure incorporates data taken from GDB (http://www.gdb.org).

2.2.4. Type X collagen

Type X collagen is a short-chain, non-fibril forming collagen molecule which is expressed in hypertrophic chondrocytes during bone growth [Schmid et al., 1987]. The molecule comprises one type of chain and exists as a homotrimer in the pericellular matrix of the hypertrophic chondrocytes. The 680 amino acid protein is encoded by the COL10A1 gene. The gene, which has been mapped to 6q22.3 [Apte et al., 1991], spans about 7kb and contains 3 exons [Thomas et al., 1991]. In order to demonstrate linkage in the present study, an intragenic point variation which introduces a *HindIII* restriction site, COL10A1, was used along with microsatellite markers, D6S474 and D6S1639 (Fig. 2-4).

The pattern of type X collagen synthesis and its distribution at the sites of cartilage calcification, suggests that it plays a role in the development of the growth plate and in the calcification of cartilage [Gordon and Olsen 1990; Kwan et al., 1991].

Heterozygous mutations of conserved amino acids in the carboxy (C)-propeptide region of COL10A1 have been identified in Schmid metaphyseal dysplasia (MCDS) [Warman et al., 1993b; Wallis et al., 1996], an autosomal dominant skeletal dysplasia characterised by short stature, short limbs and bowed legs [Dharmavaram et al., 1994]. The mutations affect the integrity of the carboxyl terminus which is responsible for the association of the collagen chains and the assembly of the triple helix [McIntosh et al., 1994; Chan et al., 1998]. Mutations associated with MCDS have, however, also been found in the genomic region coding for the N-terminal domain of the type X collagen molecule [Ikegawa et al., 1997], demonstrating the importance of this domain in the formation of the type X collagen molecule.

It was initially suggested that the MCDS phenotype was caused by haploinsufficiency demonstrating the nature and distribution of mutations within COL10A1 [Warman et al., 1993b; Jacenko et al., 1994]. However, further studies by Wallis et al. [1996] which showed a restricted distribution of 21 mutations to the carboxyl terminus of COL10A1, led these authors to conclude that haploinsufficiency alone cannot account for the MCDS disorder.

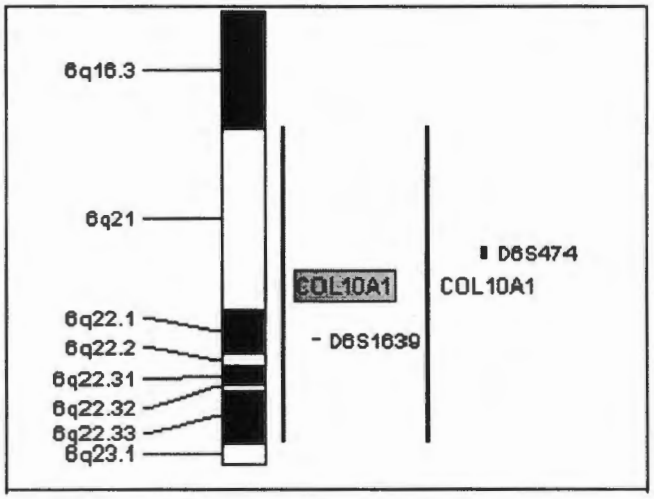

Fig. 2-4. Representation of 6q16.3-q22.3 and the approximate location of the microsatellite markers (within 10cM of the candidate region for the COL10A1 gene) which were used to show linkage to COL10A1. This figure incorporates data taken from the GDB (http://www.gdb.org).

2.2.5. Type XI collagen

Type XI collagen is a structural component of adult articular cartilage, constituting about 3% of the type II collagen content [Eyre and Wu, 1995]. The type XI collagen triple helix is a heterotrimer containing two unique gene products α1(XI);α2(XI), and also shares close structural similarities to type V collagen. The presence of hybrids containing both type XI and V collagen [Kleman et al., 1992] and the increasing production of type V at the expense of type XI during increasing developmental age [Eyre et al., 1987b] suggests that the types V and XI are isoforms of each other. Crosslinking studies indicate that type XI collagen in the cartilage matrix exists primarily as crosslinked molecules within or at the surface of type II collagen fibrils [Eyre and Wu, 1995].

The gene encoding the pro-α1(XI) chain, viz. COL11A1, has been localised to 1p21 [Henry et al., 1988], between the microsatellite markers D1S495 and D1S248 (Fig. 2-5a), and contains at least 66 exons [Ala-Kokko, personal communication]. The COL11A2 gene which was localised to 6p21.3 [Hanson et al., 1989; Kimura et al., 1989] spans 30.5kb and contains at least 62 exons [Lui et al., 1996]. Microsatellite markers within a 2% region around the gene, D1S273, D1S265 and D1S291 [GDB] (Fig. 2-5b) were selected for linkage analysis in the present study (Table 2-II). Peptide mapping revealed total similarity between α1(II) and α3(XI) chain [Burgeson et al., 1982] and it is now known that the α3(XI) chain is a product of the COL2A1 gene [Richards et al., 1996].

Type XI collagen chains copolymerise with types II and IX chains to form the cartilage collagen fibrils [Mendler et al., 1989]. It has been suggested that type XI collagen molecules limit the size of the collagen fibrils by retaining amino-terminal extensions which do not accommodate the addition of type II molecules. An increase in the ratio of type XI to type II molecules results in thinner collagen fibrils, and a decrease in the ratio results in thicker fibrils.

The absence of the α1(XI) chain in mice with an autosomal recessive chondrodysplasia confirms that this molecule is important in skeletal development. The COL11A1 mutation in the mouse model results in abnormalities in the cartilage of limbs, ribs, mandible and trachea [Li et al., 1995]. The heterozygous mutation does not manifest an obvious skeletal phenotype. A genetic defect in COL11A1 has

also been shown to be causative of autosomal dominant Stickler syndrome type 2, in which both retinal and vitreous abnormalities are present [Richards et al., 1996]. This mutation involves a Gly-Val substitution in the triple helix and has a dominant negative effect by disrupting the function of the other proteins with which the abnormal α1(XI) chain associates.

Genetic defects which have been identified in COL11A2 result in AR and AD forms of Stickler syndrome type 2 which lack the severe eye abnormalities characteristic of the phenotype produced by COL2A1 defects [Brunner et al., 1994; Vikkula et al., 1995]. This is probably due to the α2 (XI) chain in ocular tissue being replaced by α2 (V), thus overcoming the effect of the type XI abnormality. Further evidence that the α2 (XI) chain is important for skeletal development was provided by Vikkula et al. [1995] who found that a Gly-Arg substitution in COL11A2 caused an AR condition viz. otospondylomega-epiphyseal dysplasia (OSMED), which is characterised by severe degenerative joint disease in early adulthood.

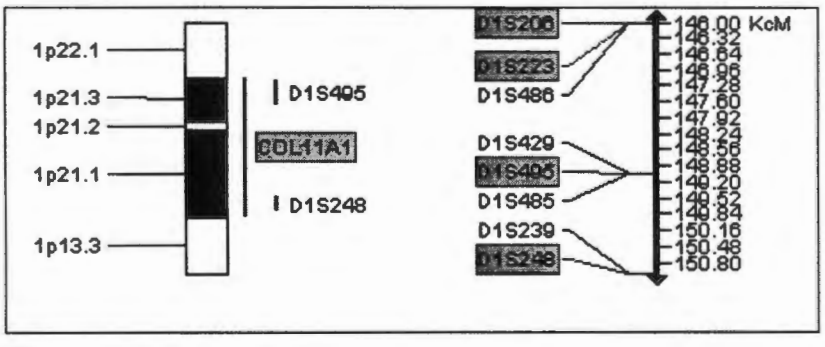

Fig. 2-5a. Representation of 1p13-p22 and the approximate location of the microsatellite markers (within 5cM of the candidate region for the COL11A1 gene) which were used to show linkage to COL11A1. The genetic distances between the markers (shaded) and relative to other markers, shown in Kosambi centimorgans (KcM), were obtained from the CEPH/Généthon linkage map. This figure incorporates data taken from GDB (http://www.gdb.org).

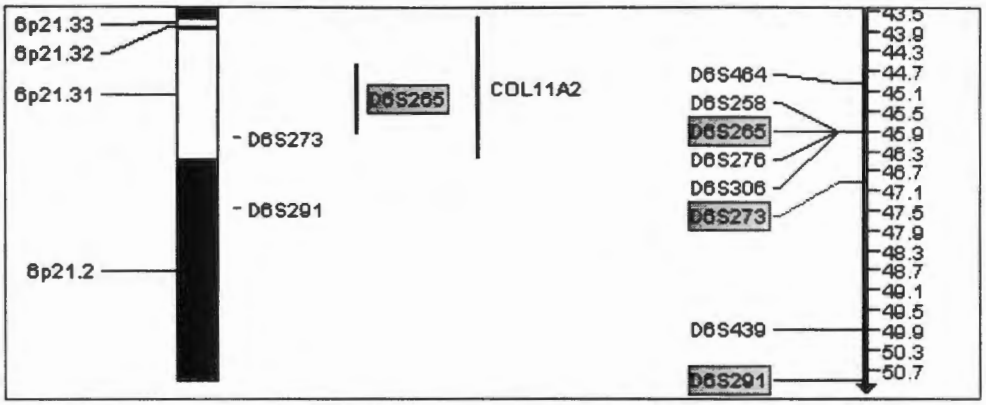

Fig. 2-5b. Representation of 6p21.2-p21.3 and the approximate location of the microsatellite markers (within 5cM of the candidate region for the COL11A2 gene) which were used to show linkage to COL11A2. The genetic distances between the markers (shaded) and relative to other markers, shown in Kosambi centimorgans (KcM) were obtained from the CEPH/Généthon linkage map. This figure incorporates data taken from GDB (http://www.gdb.org).

2.3. COLLAGEN BIOSYNTHESIS AND PROCESSING

Collagen biosynthesis requires co-ordinated transcription of genes which encode distinct monomers constituting a single protein. The process relies on multiple steps from mRNA translation through posttranslational modifications and secretion into the extracellular matrix.

A collagen molecule is synthesized as a soluble precursor, procollagen, which is translated on the free ribosomes of the RER. The procollagen polypeptide chain consists of a central helix and terminal globular domains (Fig. 2-6). The globular extensions in the procollagen molecule confer solubility and prevent aggregation and fibril assembly. The nascent chain undergoes hydroxylation of proline and lysine as well as glycosylation of hydroxylysine by enzyme modification within the cisternae of the RER. These events begin while the polypeptide chain is growing on the

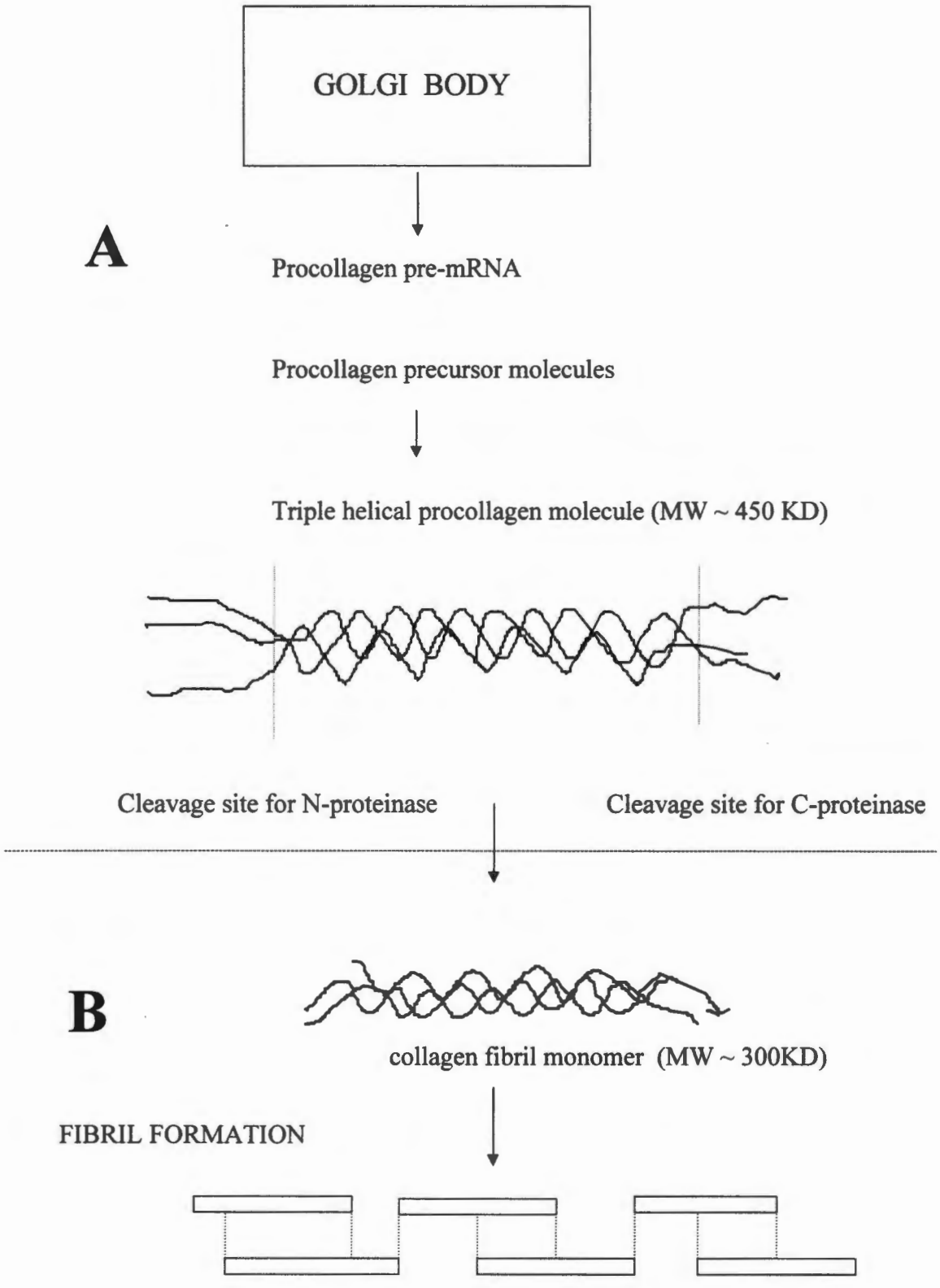

Fig. 2-6. A schematic representation of the development of the procollagen molecule indicating the intracellular (A) and extracellular (B) processes. The cleavage sites for evolution to the mature collagen molecule are indicated. (This drawing adapted from Prockop et al., 1992; Horton and Hecht, 1993)

ribosomes but continue in the form of post-translational modification until the triple helix formation is complete.

The triple helix starts forming once the procollagen monomers align themselves alongside each other. According to Ahmad et al. [1991], the integrity of the carboxyl (C) termini of the procollagen strands is essential for this alignment. The association of the C-propeptides of the 3 chains allows a 'nucleus' of triple helix to form just beyond the C-propeptide. In the triple helix the three chains are held together by a series of water and hydrogen bonds. The helix rapidly propagates in a zipper-like fashion from the C-terminus to the amino (N-) terminus. Macromolecules mix during packaging in the Golgi apparatus for transport via secretory vesicles through the cytoplasm. The triple helical molecule is readily secreted via the Golgi apparatus into the extracellular space where the amino (N-) and C- termini are cleaved off by specific N- and C- proteinases to form collagen, which spontaneously assembles itself into fibrils.

Fibril formation begins with the structuring of a nucleus by two or more collagen monomers. It then rapidly grows by the addition of more monomers to generate a large fibril. Every sub-unit of collagen that participates in fibril formation must therefore have the correct structure. Self-assembled collagen fibrils are collected into bundles and these in turn are organized into tissue specific macroaggregates that contain more than one type of collagen. In cartilage the interactions between the minor collagens, type IX and XI, with the major collagen component, type II, in the same fibril, provides a mechanism for integrating the cartilage network.

2.4. MECHANISMS OF COLLAGEN FAILURE

In both the familial and non-familial forms of OA the degeneration of articular cartilage is central to the development of the condition. A number of mechanisms can give rise to cartilage destruction; one involves external physical factors which place pressure on the joints, thus damaging the cartilage. Another mechanism involves the uncontrolled activity of proteases. Fibrillar collagens are usually stable for months or years in adult tissue and their turnover, which is triggered by injury or stress, is regulated partly by collagenases which recognise specific cleavage sites in the triple helix [Byers, 1993]. This process renders the helix susceptible to less specific proteases which can degrade the molecule further. In normal cartilage regulatory mechanisms govern the balance between the production of collagen and proteoglycan molecules, and their degradation by naturally occuring proteases. It has been hypothesized that a loss of these regulatory mechanisms could result in uncontrolled action of the proteases leading to the destruction of the cartilage matrix [Horton and Hecht, 1993]. A third mechanism involves internal factors such as mutations in genes which encode cartilage matrix components. The latter results in the incorporation of defective proteins during cartilage biosynthesis, which destabilises the cartilage matrix. Alternatively, defective proteins may result in haploinsufficiency, which is a reduction of the number of monomers available for triple helix formation and, thereby, a reduction in the total collagen content.

Prockop et al. [1992] devised experiments to determine the mechanisms by which mutations can alter the biological function of a collagen molecule. Their findings are listed below and are depicted schematically in Fig. 2.7:

(i) procollagen suicide - the C-terminus of the procollagen chain is essential for association and self assembly into collagen fibrils. However, if the C-terminus is intact but a mutation disrupts the triple helical domain of the chain, the triple helix will not be formed. The three strands will thus remain unfolded and will be degraded or secreted. In this instance some of the normal collagen chains will also be degraded since they will not be incorporated into triple helices.

(ii) persistence of the N-propeptide - if a mutation in the region of the N-propeptide prevents cleavage by the N-proteinase, the self-assembly of the collagen chains into fibrils will be either partially or completely inhibited.

(iii) base substitutions - a single-base mutation resulting in a Gly substitution results in a kink in the collagen triple helix which generates abnormally branched fibrils.

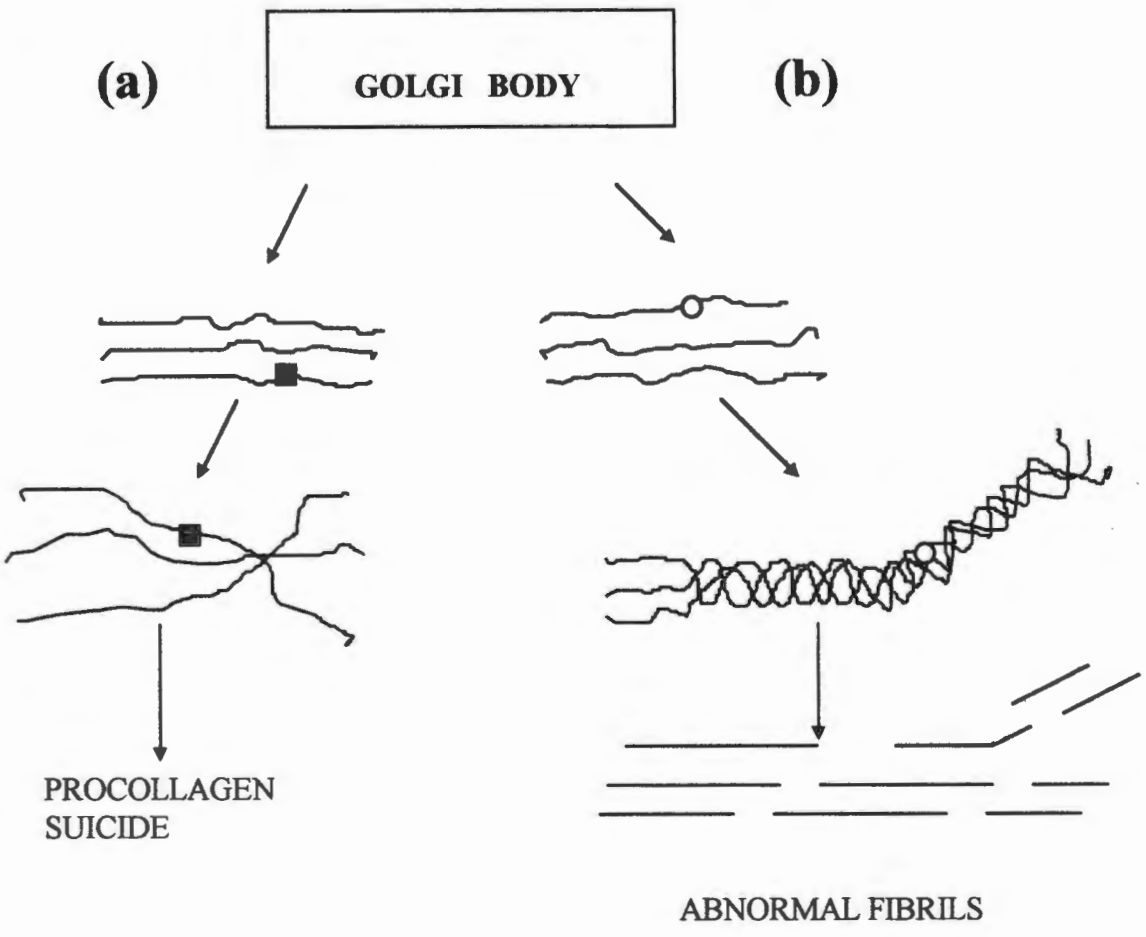

Fig. 2.7. Mechanisms of collagen failure [adapted from Prockop et al., 1992]. The drawing shows the effect of a mutation on the collagen triple helix. The mutation can either (a) result in "procollagen suicide" by inhibiting the folding of the triple helix. This results in degradation of the procollagen monomers or (b) the structurally abnormal monomer can be incorporated into the triple helix and thus result in abnormal collagen fibrils.

2.5. NON-COLLAGENOUS COMPONENTS OF ARTICULAR CARTILAGE

The non-collagenous extracellular matrix components which are all important in ensuring the normal functioning of articular cartilage include the large proteoglycan core protein, aggrecan, and the smaller proteoglycans such as decorin, fibromodulin and lumican. Other proteins included in this category are the cartilage link protein, cartilage oligomeric matrix protein, cartilage matrix protein (also known as matrilin) the chondrocalcinosis gene (CCAL2) product, and stromelysin/matrix metalloproteinase-3.

Proteoglycans are macromolecules which comprise a core protein to which a number of glycosaminoglycans (GAGs) are covalently bound. The GAGs are made up of a disaccharide repeat unit which consists of a uronic acid and a hexosamine; more than one type of GAG may be attached to the core protein. Proteoglycans appear to perform complex, but vital functions which are determined by the core protein or by the GAG chains. Through their length, the GAGs impart a high viscosity and shock absorbance to solutions, and are thus ideal lubricants in the articulating joints of the body.

2.5.1. Aggrecan

The GAG chains of aggrecan impart an osmotic pressure effect which is an important component of the total swelling pressure of articular cartilage. One of the main functions of aggrecan is its resistance to compression of tissue and its interaction with certain collagen molecules to stabilize the cartilage matrix.

The aggrecan gene, AGC1, has been localised to 15q26 [Korenberg et al., 1993] and is encompassed by the microsatellite markers, D15S130 and D15S202 [The Human Gene Map] (Table 2-II). An intragenic VNTR has been identified within the chondroitin-sulfate attachment domain of the gene [Doege et al., 1997], and was used for linkage analysis in the present study. A defect in AGC1 has been identified in murine AR chondrodystrophy (cartilage matrix deficiency) [Kimata et al., 1981; Watanabe et al., 1994, 1997]. Mice which were heterozygous for the mutation were slightly dwarfed and had vertebral abnormalities. A mutant chicken phenotype "nanomelia" has been found with the AGC1 gene [Primorac et al., 1994].

2.5.2. Cartilage link protein (CRTL1)

Stabilization of the cartilage matrix is attributable to the proteoglycan link protein (CRTL1) which is thought to bind to aggrecan and function in the early formation of proteoglycan aggregates with hyaluronic acid [Hardingham, 1979].

The CRTL1 gene which has been mapped to 5q13-14 [Osborne-Lawrence et al., 1990] has 5 exons and spans about 60kb [Dudhia et al., 1994]. A polymorphic dinucleotide repeat was used as the intragenic marker for linkage analysis in the present study. To date, the gene has not been implicated in the causation of human chondrodysplasias.

2.5.3. Cartilage oligomeric matrix protein (COMP)

COMP is a member of the pentameric thrombospondin protein family and is thought to have a calcium-binding function in the extracellular matrix. The COMP gene was localised to 19p12-13 and has 19 exons [Briggs et al., 1994; Newton et al., 1994]. The locus is flanked by the microsatellite markers, D19S49 and D19S253. Closely linked markers, D19S602, D19S212 and D19S215, were selected for the present candidate screening (Fig. 2-8).

Genetic defects in the calmodulin-like repeat domain of the COMP molecule may cause either MED [Briggs et al., 1995; Ballo et al., 1997] or pseudoachondroplasia (PSACH) [Hecht et al., 1995]. Most of the mutations involve aspartic acid residues which are thought to be essential for the calcium-binding function of the COMP molecule. Other mutations include substitutions of conserved cysteine residues in the calmodulin-like repeat region. The large-scale reduction of normal pentameric molecules in the presence of a mutated COMP monomer, suggests that these mutations may have a dominant negative effect in the extracellular matrix.

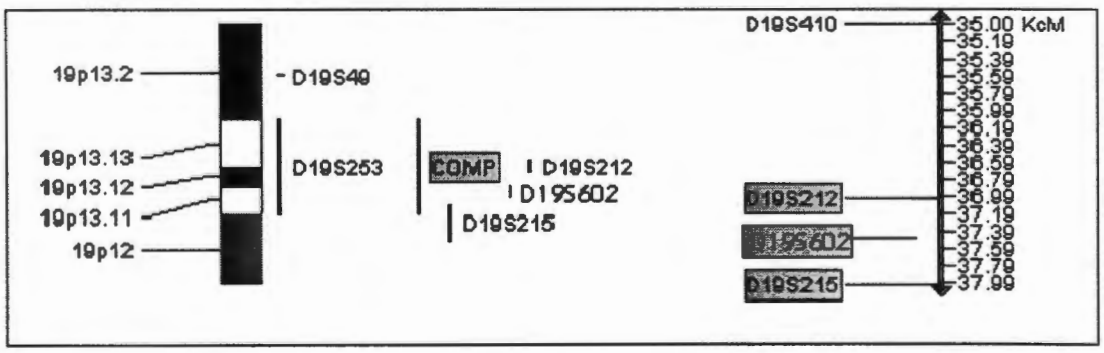

Fig. 2-8. Representation of 19p12-p13 and the approximate location of the microsatellite markers (within 5cM of the candidate region for the COMP gene) which were used to show linkage to COMP. The genetic distances between the markers (shaded) and relative to other markers, are shown in Kosambi centimorgans (KcM). This figure incorporates data taken from the GDB (http://www.gdb.org).

2.5.4. Decorin

Decorin is a small proteoglycan found in almost all connective tissues. The molecule decorates the collagen fibres, increasing the surface for interfibre interaction. The proteoglycan molecules are also thought to immobilize growth factors which they release during self-degradation and thus may play an active role in cartilage repair.

The DCN gene which was localised to 12q21-22 [McBride et al., 1990; Pulkkinen et al., 1992] between the microsatellite markers, D12S322 and D12S346 [The Human Gene Map], is larger than 38kb and contains 8 exons [Danielson et al., 1993]. Little is known about the involvement of DCN in the aetiology of chondrodysplasias.

2.5.5. Fibromodulin

Fibromodulin is a small proteoglycan which contains a core protein that is structurally related to decorin. Experimental evidence of the molecule's ability to bind type II collagen and inhibit fibril assembly, suggested that fibromodulin may participate in extracellular matrix assembly [Ayad et al., 1994].

The gene encoding fibromodulin, FMOD, is about 8.5kb in length and has been localised to 1q32 [Sztrolovics et al., 1994] between D1S1660 and D1S1678 [The Human Gene Map]. These markers along with a marker located between them, D1S306 (Fig. 2-9), were used for linkage studies. The role of the gene in the causation of cartilage abnormalities is not known.

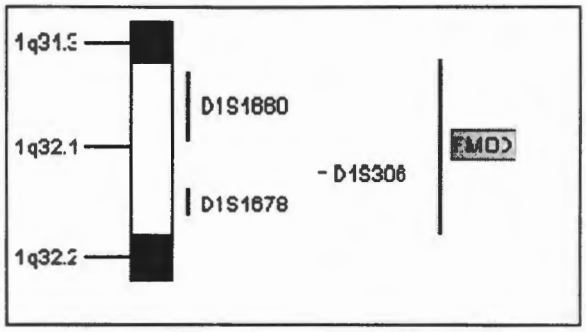

Fig. 2-9. Representation of 1q31-q32 and the approximate location of the microsatellite markers (within 10cM of the candidate region for the FMOD gene) which were used to show linkage to FMOD. This figure incorporates data taken from the GDB (http://www.gdb.org).

2.5.6. Chondrocalcinosis locus on 8q (CCAL2)

A locus on 8q has been shown to be associated with severe degenerative OA of early onset in a large family [Baldwin et al., 1995]. The condition is characterised by the deposition of calcium-containing crystals in joint tissue. Affected individuals experience bouts of acute inflammatory arthritis which is triggered by trauma, exercise or weather fluctuations. Genetic linkage results in this kindred suggest that there may be a defective gene (CCAL2) on the long arm of chromosome 8 between the microsatellite markers D8S525 and D8S284 (Fig. 2-10). Although the exact function of CCAL2 is unknown, its linkage to an early-onset form of OA suggested that it may be a suitable candidate for Mseleni joint disease. Microsatellite markers located between the flanking markers were used in the present study.

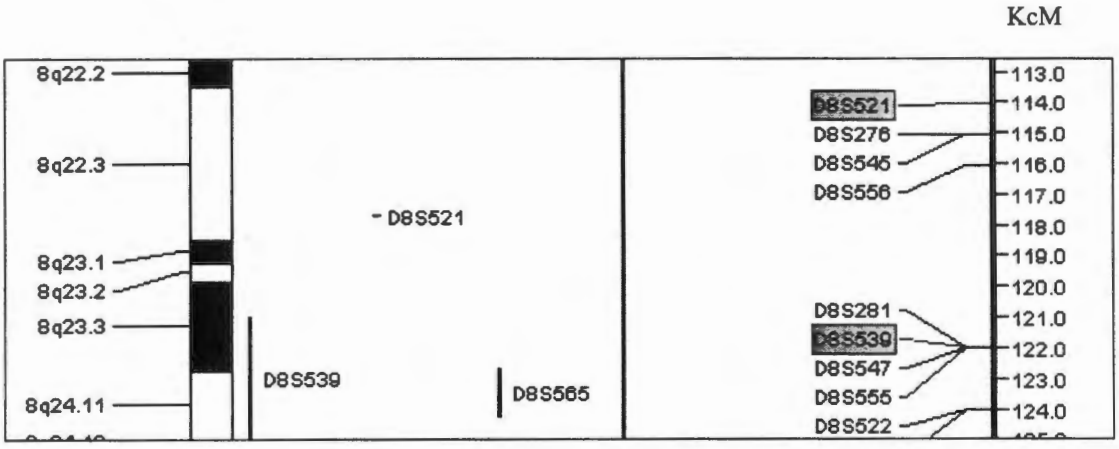

Fig. 2-10. Representation of 8q22-q24 and the approximate location of the microsatellite markers (within 5cM of the candidate region for the CCAL2 gene) which were used to show linkage to CCAL2. The genetic distances between the markers (shaded) and relative to other markers, are shown in Kosambi centimorgans (KcM). This figure incorporates data taken from the GDB (http://www.gdb.org).

2.5.7. Lumican

Lumican is a small, leucine-rich proteoglycan that is present in many tissues, including articular cartilage [Grover et al., 1995]. Similarly to the other small proteoglycans, decorin and fibromodulin, the expression is higher in adults than in juveniles.

The lumican gene, LUM, is located on chromosome 12q22 and spans a 7.5kb genomic region. Microsatellite markers within 5cM of the gene were used for linkage studies (Fig. 2-11) [Chakravarti et al., 1995]. The 3-exon gene is situated close to the DCN locus.

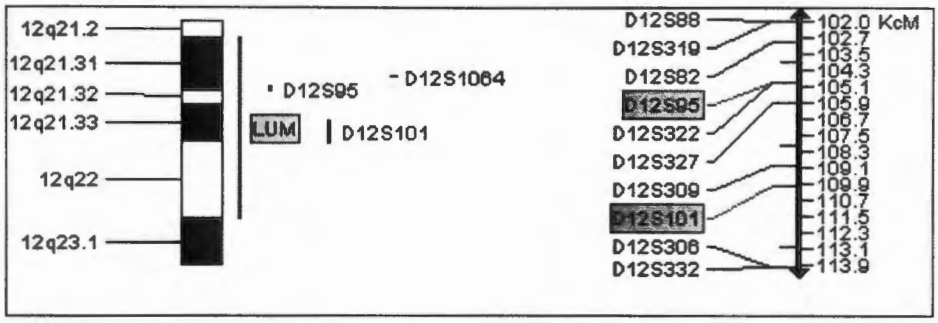

Fig. 2-11. Representation of 12q21.2-q22 and the approximate location of the microsatellite markers (within 5 cM of the candidate region for the LUM gene) which were used to show linkage to LUM. The genetic distances between the markers (shaded) and relative to other markers, are shown in Kosambi centimorgans (KcM). This figure incorporates data taken from the GDB (http://www.gdb.org).

2.5.8. Cartilage matrix protein (CRTM)

Cartilage matrix protein is a major component of the extracellular matrix protein of non-articular cartilage. The exact function of the gene, CRTM/MATN1, encoding this protein is not known, but recent investigations suggest that the CRTM locus may play a role in the sex- and joint site-specific pattern of radiologically evident OA [Meulenbelt et al., 1997]. Moreover, Okimura et al. [1997] demonstrated that articular chondrocytes can synthesize CRTM, although the synthesis is suppressed under physiologic conditions. Their results also suggest that articular chondrocytes express the protein in response to arthritic stimuli. In light of these reports, CRTM was included as a candidate in the South African study as it may well be found to play a contributory role in familial OA disorders.

The CRTM gene which spans a 12kb region on 1p35 [Kiss et al., 1989; Jenkins et al., 1990] has eight exons. A polymorphic VNTR has been isolated from the region [Wang et al., 1992] and was used in the present study.

2.5.9. Stromelysin/matrix metalloproteinase 3

Stromelysin is a proteoglycanase which is closely related to collagenase and is identical to metalloproteinase 3 (MMP3) [Wilhelm et al., 1987; Saus et al., 1988]. The gene has been localised to 11q22-q23 [Formstone et al., 1993]; the translation product is produced predominantly by connective tissue cells and is capable of degrading components of the extracellular matrix [Sellers and Murphy, 1981]. The microsatellite marker, D11S898, which is located within 5cM of the candidate region for MMP3 [GDB] was used to investigate linkage to the gene in the present study (Fig. 2-12).

Stromelysin has a high affinity for cleaving type IX collagen molecules as well as cleaving telopeptides from type II collagen, and it has the potential to degrade the connections between type II and type IX fibrils. It is therefore possible, as mentioned earlier, that if the regulatory mechanisms governing the action of these proteases are lost in a disease state such as OA, their uncontrolled action could ultimately lead to the destruction of the collagen matrix [Horton and Hecht, 1993].

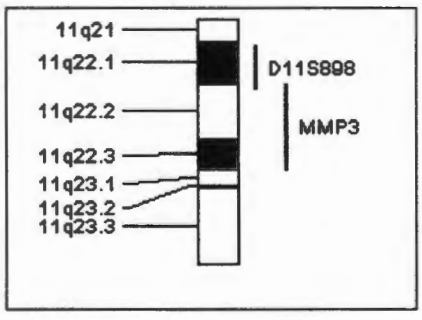

Fig. 2-12. Representation of 11q22-q23 and the approximate location of D11S898 (within 5cM of the candidate region for the MMP3 gene) which were used to show linkage to MMP3. This figure incorporates data taken from the GDB (http://www.gdb.org).

Table 2-I. The candidate genes, their chromosomal locations and the intragenic and closely linked microsatellite markers which were used for genotype analysis. The Genome Data Base is referred to as GDB and the CEPH/Généthon linkage map as CGM.

GENE	LOCUS	MARKERS	REFERENCE
COL2A1	12q13.11-12	D12S361	GDB
		D12S368	GDB
		VNTR	Stoker et al., 1985
COL6A2	21q22.3	[Ca]n	Comeglio et al., 1996
COL6A3	2q37	D2S345	GDB
		D2S338	GDB
COL9A1	6q12-13	50912B1	Warman et al., 1993
		509-8B2	"
COL9A2	1p32.3-33	MYC L1	Hellsten et al., 1995
COL9A3	20q13.3	D20S171	Brewton et al., 1995
		D20S64	CGM
COL10A1	6q22.3	VNTR	GDB
		D6S474	GDB
		D6S1639	GDB
COL11A1	1p21	D1S206	Richards et al., 1996
		D1S223	"
		D1S495	GDB
		D1S248	GDB
COL11A2	6p21.3	RFLP/*MspI*	Vikkula et al., 1995
		D6S273	"
		D6S265	"
		D6S291	"

AGC1	15q26	VNTR	Doege et al., 1997
CRTL1	5q13-14.1	VNTR	Hecht et al., 1991
COMP	19pericentro-meric region	D19S212 D19S215 D19S602	Hecht et al., 1993 Briggs et al., 1993(b)
DCN	12q21-22	VNTR	Briggs et al., 1993(a)
FMOD	1q32	D1S306 D1S1660 D1S1678	CGM GDB GDB
LUM	12q22	D12S101 D12S1064	GDB GDB
CRTM	1p35	VNTR	Wang et al., 1992
MMP3	11q22-23	D11S898	GDB
CCAL2	8q	D8S539 D8S565 D8S269	Baldwin et al., 1995 " "

SECTION II

CHAPTER 3:
A STICKLER-LIKE SYNDROME

3.1. INTRODUCTORY BACKGROUND

Stickler syndrome is a clinically and genetically heterogeneous group of autosomal dominant disorders. The classical phenotype of the Stickler syndrome is high myopia with a vitreous anomaly plus a high frequency of retinal detachment and cataracts. Hearing loss and skeletal involvement are variable and may be absent in some affected persons [Zlotogora et al., 1992]. The clinical manifestations vary between families but show little intra-familial variability. Secondary degenerative arthropathy, presumably consequent upon epiphyseal involvement, is a feature in some affected kindreds.

Different forms of the Stickler syndrome are reported to be distinguishable clinically by the presence of a vitreo-retinal component. In this regard, Snead et al. (1994) suggested that the condition can be subcategorised into type 1, in which ocular abnormalities predominate, and type 2, in which eye changes are different or absent. This concept is not, however, universally accepted. In any event, it is likely, that there is even greater heterogeneity.

Stickler syndrome type 1 has thus far been found to be associated with the COL2A1 gene which encodes type II collagen [Snead et al., 1994]. In all affected persons investigated, the causative gene defect has been shown to be a termination signal leading to premature truncation of type II procollagen chains. Seven of the 9 mutations described in affected individuals have been single base changes (Table 3-I) resulting in premature stop codons and truncated polypeptide chains which impair the assembly of the procollagen triple helix [Ahmad et al., 1991, 1993, 1995; Brown et al., 1992, 1995; Ritvaniemi et al., 1993]. Either an in-frame stop codon is created or the reading frame is shifted so that an out-of-frame stop codon is encountered downstream. The resultant polypeptide chains are truncated and lack the noncollagenous carboxy -propeptide which is necessary for incorporation into triple-helical molecules. It is predicted that type II collagen triple helices are reduced in the cartilage matrix (haploinsufficiency) which can therefore not function properly as a template for endochondral ossification [Horton, 1995]. In Stickler syndrome the type II collagen defect manifests in the eye since the protein is a major component of the vitreous humour. The reduction in type II collagen possibly results in an abnormality of the interface between the retina and the vitreous jelly, resulting in the retinal detachments seen in Stickler syndrome.

The other reported mutations involve a 10bp insertion [Brown et al., 1995] and a 16bp deletion [Williams et al., 1996] which result in termination signals further downstream and consequently truncated peptides. In Stickler syndrome type 2, ocular involvement is either different from that in type 1, or entirely absent [Snead et al., 1994]. To date, this form of the disorder has not been linked to COL2A1, but the phenotypes in two large families have been associated with COL11A1 [Richards et al., 1996] and COL11A2

[Brunner et al., 1994]. There are, however, some kindreds in which the disorder has not been linked to either the COL2A1 or COL11 loci, thereby demonstrating additional non-allelic heterogeneity [Knowlton et al., 1989; Vintiner et al., 1991; Bonaventure et al., 1992].

In the present study, a South African family of Afrikaner stock, with early onset myopia, conductive deafness and generalised epiphyseal dysplasia was investigated for a molecular defect. The affected relatives were initially described by Beighton et al. [1978] and reviewed again in 1994. At this time, and with informed

Table 3-I. Termination mutations in the COL2A1 gene which result in Stickler type 1 syndrome.

EXON	MUTATION	POSITION OF STOP CODON	REFERENCE
40	1 bp substitution	Arg 732	Ahmad et al., 1991
40	1 bp deletion	Exon 42	Brown et al., 1992
7	1 bp substitution	Arg 9	Ahmad et al., 1993
43	1 bp deletion	Exon 44	Ritvaniemi et al, 1993
4	10bp insertion	Exon 5	Brown et al., 1995
20	1 bp deletion	Exon 26	Brown et al., 1995
48	1 bp insertion	Exon 51	Brown et al., 1995
50	1 bp deletion	Exon 51	Ahmad et al., 1995
18	16 bp deletion		Williams et al., 1996

consent, venous blood specimens were obtained for the molecular studies which form the subject of this chapter. These molecular findings have been published in an article entitled "A Stickler-like syndrome due to a dominant negative mutation in the COL2A1 gene" [Ballo et al., 1998].

3.2. METHODS AND MATERIALS

(i) The Family

The affected family belongs to the Afrikaans-speaking Caucasian community of South Africa. Family members were ascertained in 1978 when three affected sibs were encountered during diagnostic surveys in special schools for the deaf in the city of Pretoria, South Africa. Detailed otological, ophthalmological and radiographic studies showed that these children had myopia, bilateral conductive deafness, a round flattened facies and mild generalised epiphyseal changes. Crenated cataracts and asteroid hyalosis of the vitreous were variable manifestations in the affected individuals. The condition was regarded as a form of multiple epiphyseal dysplasia (MED) and in particular, the joint changes and stubbiness of the digits were in keeping with this diagnosis. The clinical and radiographic features of the affected persons are depicted in Fig. 3-1 and Fig. 3-2a. Their mother was similarly affected, but another brother, and the mother's parents and sibs were normal. Pattern profile analysis using the method developed by Poznanski et al. [1972], was undertaken to determine the extent of digital shortening (Fig.3-2b). At the clinical level, the unaffected family members did not have stunted digits.

Fig.3-1. The clinical features of the affected kindred. The affected mother and her three affected offspring who all have high myopia, stubby digits and a round flat face. (From Beighton et al., 1978. Reprinted by permission of the editor of Clin Genet).

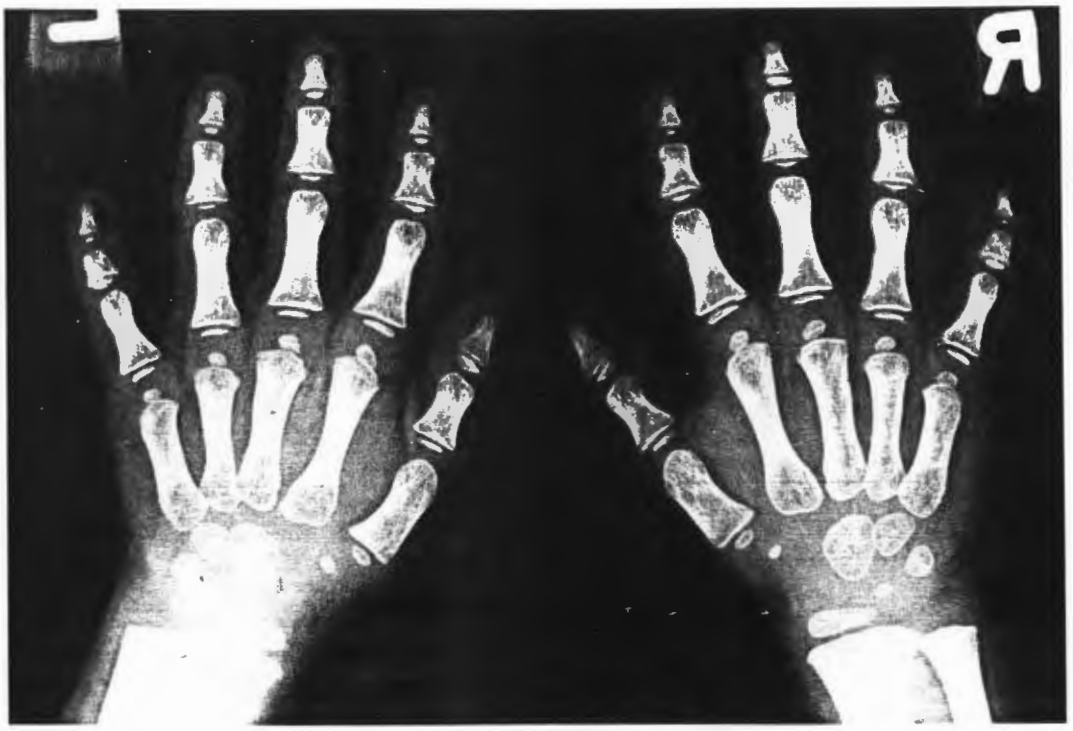

Fig. 3-2(a) Stickler-like kindred: Anterior-posterior radiograph of the hands of an affected child, showing the short phalanges.

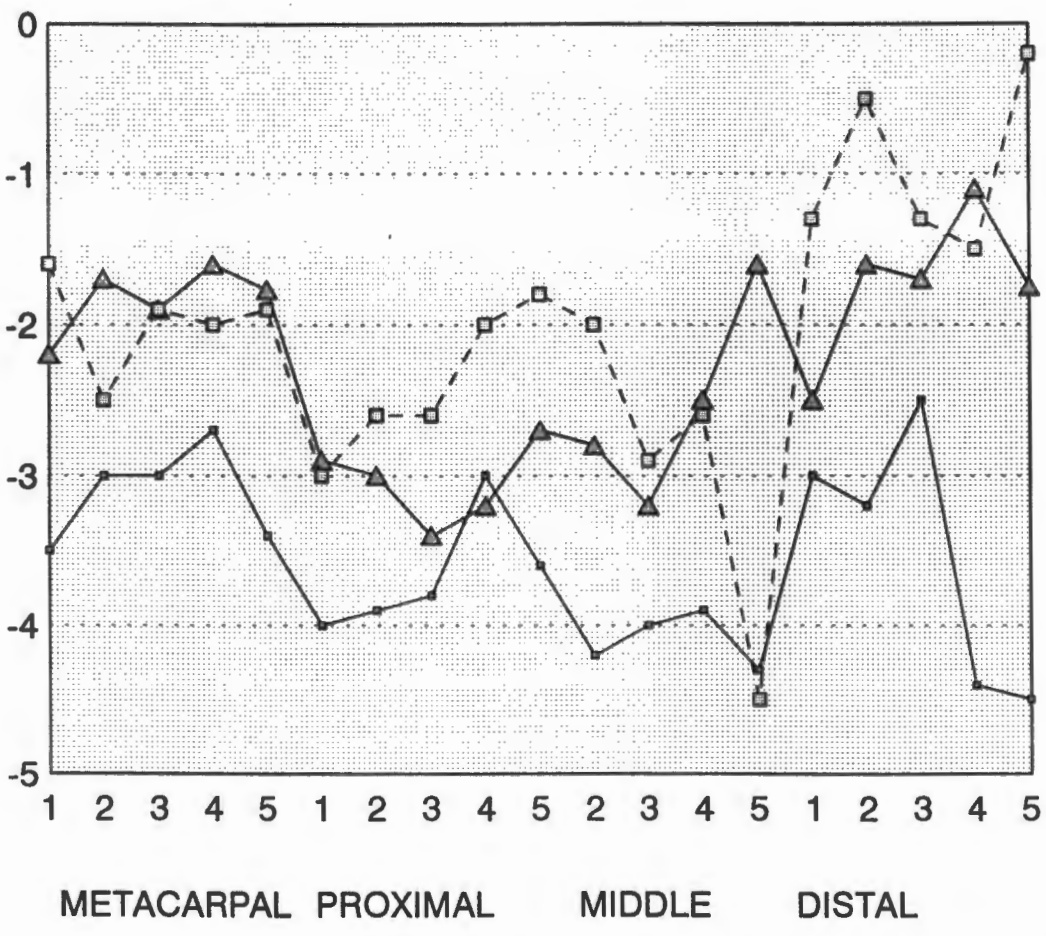

Fig.3-2(b). Stickler-like kindred: Pattern profile analysis of the digits of the proband age 33 (M33) and two affected children aged 6 (F6) and 7 yrs (M7). Shortness of all the tubular bones is evident.

(ii) Molecular Genetic Investigations

Linkage Analysis

Peripheral venous blood (10ml) was obtained by venipuncture. Genomic DNA from family members was extracted using the Genomix™ blood scale-up kit [*Talent*, Italy](see Appendix 1, section 1.1.). The COL2A1 chromosomal region, 12q13.11 - 12q13.12 [Takahashi et al., 1990], was genotyped using primers for the COL2A1 3' VNTR [Stoker et al., 1985] and the microsatellite markers, D12S368, D12S361 and D12S339 [*Research Genetics*, USA]. The localisation of these markers on chromosome 12 is given in Ch.2, section 2.2.1. In addition, markers closely linked to the MED candidate loci, EDM1/COMP (D19S215 and D19S212 - see Ch.2, section 2.5.3.) [Briggs et al., 1995] and EDM2/COL9A2 (MYCL1 - see Ch.2, section 2.2.3.) [Warman et al., 1994; Hellsten et al., 1995] which were commercially available [*Research Genetics*, USA], were also tested.

The PCR reactions were performed with 100-200ng of genomic DNA as templates; 5-10pmol of each primer; 1μCi of α-[32P]dCTP; 200μM dATP, dCTP, dGTP and dTTP; and 0.5U of *Taq* polymerase [*GibcoBRL*, UK], in a final volume of 10μl. Samples were cycled 25 times in a Omnigene [*Hybaid*, UK] PCR machine using annealing temperatures of 60°C (COL2A1 VNTR) or 55 °C (microsatellite markers). PCR products were checked for size by comparison with a 1kb molecular weight marker [*GibcoBRL*, UK] on 2% agarose [Sigma, USA] gels. Ten μl of formamide loading buffer (Appendix 1, section 2.1.2.) was added to each reaction after PCR. The samples were denatured for 3 minutes at 95°C and chilled on ice for 10mins, followed

by electrophoresis through 6% denaturing polyacrylamide gels (see Appendix 1, section 2.1.3). The gels were dried onto 3MM paper [*Schleicher and Schuell*, Germany] on a slab gel drier [*Hoefer Scientific Instruments*, USA] for 1-2hrs at 80°C and exposed to X-ray film [*Agfa*, Germany] for 16-32hrs at -70°C. The autoradiographs were developed and the alleles scored for the microsatellite markers.

Linkage analyses were performed using the LINKAGE package Version 5.03 [Lathrop and Lalouel, 1984] and the map order from the Généthon map of microsatellite markers for each chromosome. In all analyses the number of alleles for each marker was reduced to five, without loss of information in the family, in order to accommodate the computer program's limitations for numbers of alleles. Male and female recombination rates were taken to be equal. The condition was assumed to be autosomal dominant with full penetrance and a gene frequency of 0.001 was assumed for the disease locus.

Mutation Analysis

Once the determinant gene had been identified using the linked microsatellite markers, the exons of the gene were screened for small mutations using SSCP analysis (see Appendix 1, section 2.2.1). Genomic DNA was extracted from blood samples and the exons and flanking sequences of the triple helical domain were amplified by a series of intronic primers (Table 3-II). The PCR was performed in a total volume of 25µl using primer-specific annealing temperatures (Table 3-II). PCR products were checked for size by comparison with a 1kb molecular weight marker [*GibcoBRL*, UK] on 2% agarose [*Sigma*, USA] gels. Formamide loading buffer (see

Appendix 1, section 2.2.1.2.) was added to each reaction in a 1:1 dilution, the samples denatured for 2 mins at 95°C and then chilled on ice for 5mins to allow the single stranded DNA to reanneal. Ten µl of the sample was electrophoresed through a non-denaturing 0.5 x MDE™ gel matrix [*FMC BioProducts*, USA] at 5-10W for 18 hrs followed by silver staining to visualize the DNA banding patterns [Lohmann et al., 1992]. The methods were as decribed in Appendix 1 (section 2.2.1.2).

Following the observation of SSCPs, the relevant exon was sequenced in order to determine the nucleotide changes which resulted in the bandshifts. The double-stranded PCR products were purified using the QIAquick protocol for eluting DNA from a gel matrix [*Qiagen*, Germany] and sequenced, in the 5' and 3' directions, using the Sequenase version 2.0 DNA sequencing kit [*United States Biochemical (USB)*, USA] (see Appendix 1, section 2.2.2). Sequencing reactions were analysed on 6% PAGE gels, dried at 80°C and visualised by autoradiography.

Table 3-II. Oligonucleotide sequences of primers used to amplify the exons of the triple helix of the COL2A1 gene.

Exon	PCR Primer sequence	PCR product (bp)	Annealing temperature (°C)
6	5'-GTGCCTTTCAACCTCCTAACG-3' 5'-CCTGCCATTTTGGCTGCAAAG-3'	157	57
7/8	5'-GCATGTGTAGTAAACCCCTC-3' 5'-GGACAGGGCCGTGCTGGTAC-3'	272	55
9	5'-CTATAACCATCTCTTAAACTATC-3' 5'-AGGAGGCAGCTCCTCATTTG-3'	131	55
10	5'-GTCTCTGAGGAAGCTGGGATA-3' 5'-CTAGGAAAGATGCCTGAGGCT-3'	132	59
11	5'-CTCTGTGCTCTGAACACCTCC-3' 5'-GGCATCTCTTCCTTCCAACC-3'	128	57
12	5'-GTAAGTATCACGGGTGAGAAG-3' 5'-GCTAGTTCCACTGAGCTCCAC-3'	282	57
13	5'-GTGGAGCTCAGTGGAACTAGC-3' 5'-GAGTCTTTGATAAACCTTCCTGG-3'	312	60
14	5'-AGCCAACTCATGCTTAGGCTG-3' 5'-AATGGTGGTGTTTGGCTTTGTC-3'	124	59
15	5'-CTGTGGGCACCTCTCATGG-3' 5'-GCACAGCAACAATGACCTGCT-3'	124	57
16	5'-CTGGCATTCACCTCTCTTCTC-3' 5'-GCACCCAGAAGTTCCTGACTG-3'	131	55
17	5'-CTTCATTTCATACCTCCCTGTC-3' 5'-CCCAGGCCAAAGAGAAGCTG-3'	156	59
18	5'-GCTGCTTATTTGACAATGTCTC-3' 5'-GGCTCTCCTGGGGTAGCAAAG-3'	114	57
19 (*1)	5'- CTGTGAGTGTTGCCCGTGGAC -3' 5'- CCAGAACCCCTGTTCAAGATG -3'	325	60

20	5'-GGAGAGAATCTGGTGTGAGG-3' 5'-CAGCAGCAGAGAAGACAAGG-3'	132	57
21	5'-GTGAAGGCTCACTCTGTTTC-3' 5'-CCATGGGATGGAGCCTCCAC-3'	178	56
22	5'-CTGTTCTCACTCACTGCCTC-3' 5'- AGAGAAGAGGGTGGGGTCAG-3'		57
23	5'-CTGACTCCCTGTGTACCCTTG-3' 5'-AGAGAAGAGGGTGGGGTCAG-3'	248	55
24	5'-CAGCCCTGCACTGCCAGGAT-3' 5'-CCCTCCTAGCAGCCCTCAGC-3'	160	60
25	5'-GTGTCTATGTTCTGAGAATGGTG-3' 5'-CACCACATGGAAGGAAATAGAAG3'	174	59
26	5'-AACCATCCGCCTCATGGCC-3' 5'-GGCCAACACCAAGTCATGGG-3'		57
27/28	5'-AAGCGTGTCATTGGACTTTTCTT-3' 5'-TGGCCCCCAGGGCCACCTG-3	692	55
29 (*1)	5'- GTCTGCCCTATACTGTGCCTC -3' 5'- ATGCCCTCTTGCCCTTGCCTC -3	217	61
30/31 (*2)	5'-TCTGTGATATGAAGCCTTCACTC-3' 5'-ACTGGATGCAGCCTCACTTA-3'	358	61
32	5'- GGAGCTGGAAAGGAGGTCTCA -3' 5'- GGTGCCATAAGGGAACGGAAG -3'	329	61
33	5'-GTGCCCGGCTGAGGCGGCTG-3' 5'-TCCTAATGCCCAGCAGTCCAG-3'	130	55
34	5'-CTCAGCCTGCCTCCCTCACC-3' 5'-GGGAGGCAAGGTGTGGAGAG-3'	133	55
35	5'-ACCTTCTTACCCCAGCTCTTC-3' 5'-GGCCTCGGGCAGAGCCAGGC-3'	134	60
36	5'-CAGCGGGGCCTGACTCTCGC -3' 5'-CTGAGTGGAGGTACCCAGGAG-3'	134	56

37	5'-CACCTGCTTCCTCCTTCCCCA-3' 5'-AATTCTTGGAGTGCAGCGTTAC-3'	161	55
38	5'-GCCACTGGGCCTCACTGTC-3' 5'-TTTGTGAGGTGCAGGGTGGG-3'	131	59
39	5'-AGGGCTTGAGGTTCTCAGGG-3' 5'-CATGCCTGCCTGTGCCTCTC-3'	134	55
40	5'-GGTGAGATGAGTCCTCACTTC-3' 5'-GGCATGGGCCTGGTGAGGG-3'	227	55
41	5'-GGCCACTGTCAGTTCTCATCTC-3' 5'-AGGGCCAGCTTGGATGGAGG-3'	188	55
42	5'-CACTCATCATCCTTGTCTCTGTC-3' 5'-CTCCCCAATCAGGGCCACCC-3'	177	55
43	5'-GGAAGCAGCTCTAAGTGCATTC-3' 5'-CACTGCACACACAGACACCAG-3'	123	55
44	5'-GGAACATTCTTCTCTGAGCCTG-3' 5'-GACGTTGTAAAACGACGGCCAG-3'	188	55
45	5'-TCCTGGCTTTTCTCTGACGCTG-3' 5'-TCCCTGGTGGGGACTCAGTG-3'	134	55
46	5'-CCCTGACCTGACTCAATCGG-3' 5'-AGGAGGCCTCGGGAAGTCCC-3'	185	55
47	5'-GCTGACCGTGGCCTTTTGCC-3' 5'-AAGGGCCCCCTCCATCTTCC-3'	127	55
48	5'-CCAACTTAGGGTTCCATGACTG-3' 5'-GGGACACCTCGACAGCAGGG-3'	291	57
49	5'-CACACAATCCTGGCTGATCTC-3' 5'-CCTGCTTCCCGGGGCAGG-3'	366	57

NOTE- For each exon the forward or sense primer is given on the top line and the reverse (antisense) primer is given below. DNA was amplified under the following PCR conditions: denaturation at 94°C for 30 secs, annealing temperature as tabulated and extension at 72°C for 30 secs. Primer sets marked with *1 were obtained from Dr Jarmo Korkko, PA, USA. Primer sets marked with *2 were were designed in the UCT laboratory. The remaining primers were obtained from DJ Wilkin and DH Cohn, LA, USA.

3.3. RESULTS

The pedigree of the portion of the family which was available for investigation is shown in Fig. 3-3(a). DNA from individuals I-1, I-2, II-1, II-2, II-3, and II-4 was available for linkage analysis. Recombinant meioses, detected with the markers D19S215 and MYCL1, led to the exclusion of COMP and COL9A2. Taking into account the size of the available family, the lack of recombination and LOD scores of 0.90 (D12S361), 0.90 (D12S339) and 0.86 (D12S368) at theta=0.00 provided evidence against the exclusion of the candidate gene, COL2A1, and led to its further investigation.

A conformational polymorphism in an amplified DNA fragment containing exon 39 of the COL2A1 gene consistently cosegregated with the condition in the family (Fig. 3-3b). Neither the unaffected family members nor 54 normal random controls from the South African Afrikaner population, exhibited a variant SSCP pattern. DNA sequencing revealed a C2503T transition (Fig. 3-4), implying the substitution of a basic arginine by an uncharged cysteine residue at position 704 of the type II collagen molecule.

3.4. DISCUSSION

The South African family in this study has a condition which resembles Stickler type 1 syndrome, the only non-characteristic feature being brachydactyly. The affected

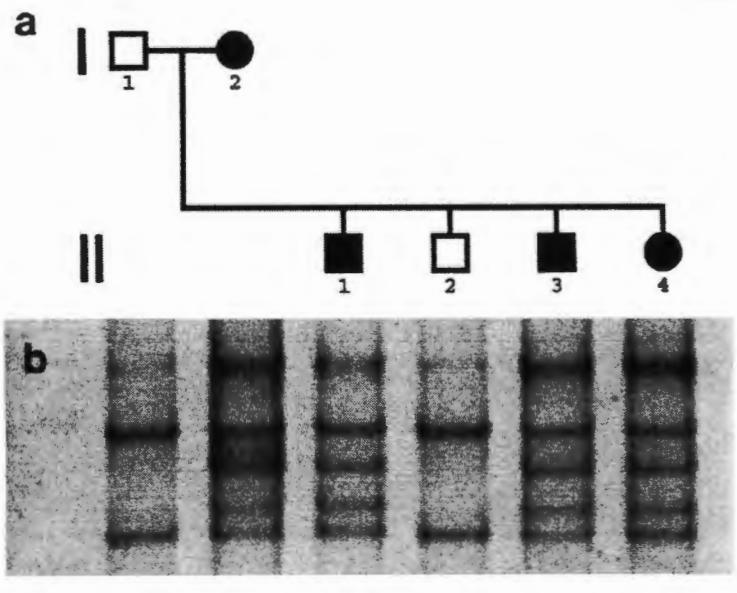

Fig.3-3. Stickler-like kindred: (a) Pedigree and (b) PCR-SSCP analysis of exon 39 of the COL2A1 gene in a South African kindred with a phenotype resembling Stickler type 1 syndrome. Shaded symbols on the pedigree represent affected individuals. The SSCP change cosegregates with the disorder.

a.

nt position 2503

GGA GCT GCT GGC CGC GTT GGA
Gly Ala Ala Gly **Arg** Val Gly

aa position 704

GGA GCT GCT GGC TGC GTT GGA
Gly Ala Ala Gly **Cys** Val Gly

b.

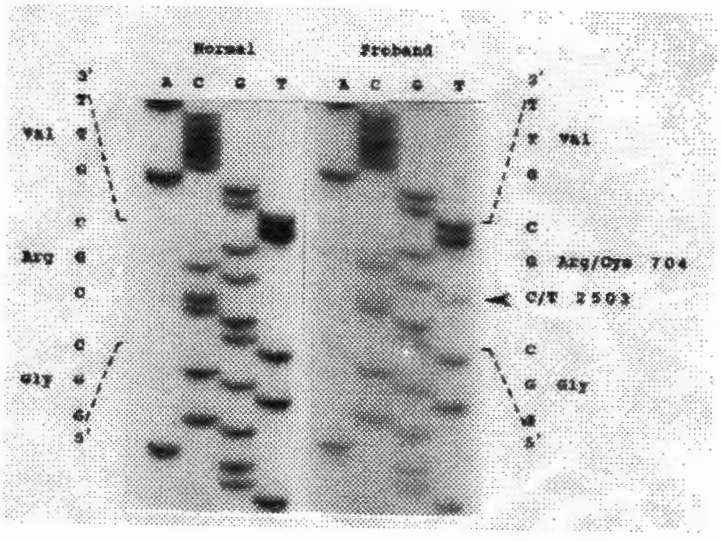

Fig. 3-4. Sequence analysis of normal and mutant PCR-amplified genomic DNA. (a) Partial sequence of COL2A1 exon 39. The normal and abnormal alleles are shown. (b) Results of sequence analysis. The sequencing gel showing the region of exon 39 of the COL2A1 gene in which the base substitution occurs. The arrow indicates the C-T transition at nucleotide (nt) position 2503.

individuals were initially described and depicted in an article entitled "Dominant inheritance of multiple epiphyseal dysplasia, myopia and deafness" [Beighton et al., 1978]. The phenotype, however, closely resembled that of Stickler syndrome type 1 and the issue of diagnostic categorisation remained a matter of concern.

The nature of the COL2A1 mutation identified in this kindred, i.e. the arginine to cysteine conversion, has previously been associated with a spectrum of spondyloepiphyseal dysplasia (SED) phenotypes (Table 3-III), whereas Stickler type 1 syndrome has only been associated with termination mutations. In order to make an accurate classification of the clinical phenotype in this kindred, it may therefore be necessary to take into account the broad spectrum of type II collagenopathies which can be caused by a defective COL2A1 gene.

The term SED is applied in the broad sense to any chondrodysplasia in which the vertebral bodies and the epiphyses of the long bones are primarily involved. In accordance with this definition, Stickler syndrome can be included in this wide category. In the narrow sense, classical SED is conventionally subdivided into the autosomal dominant SED congenita [SEDC] and the X-linked tarda [SEDT] forms, both of which have specific clinical and radiographic phenotypes. However, there is considerable variability and several other forms of SED have been delineated, the phenotypic spectrum ranging from severe dwarfing dysplasias with facial, ocular and auditory abnormalities through to mild epiphyseal dysplasias with premature osteoarthritis [Cole et al., 1994].

The condition in the South African family described in this study resembles that of the family described by MacDermot [1987], the main features of both conditions being myopia and sensorineural deafness. Although the 'MacDermot' phenotype resembled both Stickler type 1 syndrome and SED congenita (SEDC), it was considered to be a distinct entity. With regard to the brachydactyly component, the only other report of SED with brachydactyly, concerned a family from the Chiloe islands off the southwest coast of South America, who had a form of SED Tarda (SEDT); their other features included precocious OA and intra-articular calcification [Reginato et al., 1994]. The affected Chiloan islanders had no ocular abnormalities while their severe skeletal problems necessitated hip replacements; conversely, skeletal involvement in the South African kindred was of a relatively minor degree.

The gene encoding type II collagen, COL2A1, has been implicated in a number of disorders in the general SED category, including some, but not all, instances of the Stickler syndrome. There is further semantic complexity as COL2A1 has been found to be involved in "mild chondrodysplasia with osteoarthritis" [Bleasel et al., 1995] and "SED and precocious osteoarthritis" [Williams et al., 1993]. These disorders are not autonomous entities as degenerative osteoarthropathy can be a complication of virtually any form of SED. Type II collagen has been shown to be defective in some instances of Stickler syndrome type 1 [Snead et al., 1994] which is typically characterised by severe ocular abnormalities with other manifestations such as hearing loss and epiphyseal dysplasia. The condition is, however, clinically and

Table 3-III. Chondrodysplasia-causing Arg-Cys mutations which have been identified in the type II collagen triple helix.

LOCATION	EXON	POSITION IN GLY-X-Y	PHENOTYPE	REFERENCE
Arg75 - Cys	11	Gly-Ala-Arg	SED with precocious OA No ocular abnormality	Williams et al., 1993 Reginato et al, 1994
Arg519 - Cys	31	Gly-Pro-Arg	Precocious OA with mild chondrodysplasia No ocular abnormality	Ala-Kokko et al., 1990 Eyre et al., 1991 Holderbaum et al., 1993 Williams et al., 1995b
Arg789 - Cys	41	Gly-Gln-Arg	SED congenita Variable myopia	Chan et al., 1993a
Arg704 - Cys	39	Gly-Arg-Val	Stickler-like syndrome Myopia and deafness	Present study, Ballo et al., 1998

genetically heterogeneous and there is considerable controversy concerning the syndromic boundaries and nosology of the Stickler syndrome. The South African family reported in this study has a phenotype which is compatible, to some extent, with the Stickler syndrome although the digital shortening in the affected persons is not in keeping with this diagnosis.

The COL2A1 mutations thus far described in individuals affected with Stickler syndrome type 1, have been mostly single base changes. All of the COL2A1 mutations causing the disorder result in premature stop codons and truncated polypeptide chains which lack a carboxyl terminus [Ahmad et al., 1991, 1995; Brown et al., 1992, 1995; Ritvaniemi et al., 1993]. Since this domain is essential for the initiation and propagation of the type II collagen triple helix, it is likely that the defective chains are not selected for assembly of the trimeric molecule [Ahmad et al., 1991]. It was previously postulated that a mutation which does not allow an abnormal molecule to be incorporated, or one that reduces the amount of collagen chain monomer available for incorporation (haploinsufficiency), would be expected to have mild consequences [Sykes et al., 1983]. Thus, in Stickler syndrome type 1, because the carboxyl-deficient procollagen chains would not be included in the triple helix, their interference during fibril formation would be minimal.

In the South African family the COL2A1 mutation involves the substitution of a basic arginine residue at amino acid position 704, by an uncharged polar cysteine residue. Arginine-cysteine conversions are not novel to the COL2A1 triple helix which appears to have 'hotspots' for these changes [Table 3-III]. Possibly, the incorporation

of cysteine, which leads to crosslinking with other cysteine residues, results in abnormal procollagen molecules which are less stable and are therefore secreted less efficiently from the RER of the cell [Byers et al., 1991]. Furthermore, the abnormal molecules which are incorporated into the cartilage matrix interfere with the normal interaction between triple helices and other matrix components during fibril formation. In previous studies, biochemical investigations of the cartilage from persons with an Arg519Cys and Arg789Cys substitution have shown overmodified α1[II] chains which formed abnormal disulfide-bonded dimers and trimers [Eyre et al., 1991; Chan et al., 1993a]. Several unrelated persons with a mild form of SED have been shown to have cysteine substitutions at either Arg75 [Williams et al., 1993; Reginato et al., 1994; Bleasel et al.,1995] or Arg519 [Ala-Kokko et al., 1990b; Holderbaum et al., 1993; Pun et al., 1994; Williams et al., 1995]. Other studies, [Chan et al., 1993b] described individuals with the more severe SEDC and who were heterozygous for an Arg789Cys mutation. The increased phenotypic severity in individuals with the Arg789 substitution is in keeping with the proposed gradient model which relates severity of skeletal abnormalities with the position of the mutation within the collagen triple helix [Horton et al., 1996]. There are, however, inconsistencies within this model and it is becoming evident that molecular findings cannot provide a definitive basis for the categorisation of all of the type II collagenopathies. Exemplifying this observation is the South African family in this study, which, by virtue of the position of the substituted amino acid, would be expected to have a severe skeletal dysplasia [Bächinger et al., 1993], but which in fact has very mild skeletal abnormalities.

The triple helix of the collagen molecule consists of a Gly-X-Y repeat in which X and Y can be any amino acid except cysteine and tryptophan [Byers et al., 1991]. In the South African family the relatively mild phenotype, which differed from more severe clinical manifestations observed in the other SEDC patients with Arg-Cys changes, can probably be ascribed to the position of the substituted amino acid within the Gly-X-Y repeat. In all of the other Arg-Cys conversions, the arginine residue occupies the Y position of the Gly-X-Y triplet repeat. The mild skeletal involvement observed in the South African family may be explained by the fact that the substituted nucleotide occupies the X position in the Gly-X-Y repeat. Although substitutions in the Y position may be tolerated, resulting in mild SED phenotypes [Byers et al., 1991], it is possible that substitutions in the X position may be even less deleterious; the X position probably is not as exposed to intra- and interhelical interactions as the Y position which sits on the outside of the triple helix [Chan et al., 1993]. Thus, although the substitution occurs close to the carboxyl terminus of the procollagen chain and would therefore be expected to cause a severe SEDC phenotype [Bächinger et al., 1993], in actual fact the affected persons in the South African family have relatively mild skeletal problems. In this context the findings of this study may contribute to an understanding of genotype-phenotype correlations.

The COL2A1 Arg-Cys mutation identified in this kindred underlies an unusual type II collagenopathy. This mutation could, however, also represent the first report of a non-termination COL2A1 mutation in Stickler syndrome type 1, the first report of a mild SEDC, or the first report of a COL2A1 defect in an MED phenotype.

CHAPTER 4:
SPONDYLOEPIPHYSEAL DYSPLASIA (SED): SED TYPE CAPE TOWN (SEDCT) AND NAMAQUALAND SED (NSED)

4.1. INTRODUCTORY BACKGROUND

The spondylo-epiphyseal dysplasias (SED) are a clinically and genetically heterogeneous group of disorders characterised by abnormalities of growth and development of cartilage and/or bone. Classical SED can be subdivided into two forms: the autosomal dominant SED congenita (SEDC) and the X-linked recessive form, SED Tarda (SEDT). SEDC is characterised by clinical and radiological stigmata which are present at birth whereas SEDT has its onset in mid-childhood. In addition, the SED category encompasses a range of atypical phenotypes in which abnormalities of the spine and the ends of the long bones predominate [Horton, 1996; Rimoin, 1996].

The most likely candidate for the causation of SED is the COL2A1 gene which encodes type II collagen, the major component of articular cartilage. COL2A1 mutations have been identified in a variety of cartilage disorders which constitute a clinical spectrum of skeletal dysplasias, many of them falling into the broad SED category. These disorders range from mild conditions such as Wagner syndrome and the Stickler syndrome type 1, through to the more severe Kniest dysplasia, spondyloepimetaphyseal dysplasia type Strudwick (SEMD), SED congenita (SEDC)

and the lethal achondrogenesis and hypochondrogenesis disorders (Table 4-I) [Kuivaniemi et al., 1997]. The most common mutations resulting in the SED phenotypes involve single base mutations which substitute the glycine residue in the Gly-X-Y repeat with a bulkier amino acid (Table 4-I).

Early biochemical studies of cartilage from persons with various forms of chondrodysplasia provided evidence of changes in the collagen triple helix which relate to a gradient of phenotypic severity; generally, mutations occuring close to the carboxyl terminus result in the more severe chondrodystrophies whereas in milder phenotypes, mutations occurred closer to the amino terminus [Chan et al., 1991; Horton, 1996]. Some mutations do not, however, fit the gradient pattern. For instance, Gly895Ser, causes a SED phenotype although a severe chondrodysplasia would be expected because of its proximity to the carboxyl terminus [Winterpacht et al., 1995].

This chapter describes investigations on familial SED which has been encountered in two large kindred in the South African mixed ancestry population, viz. SED type Cape Town (SEDCT) and Namaqualand spondyloepiphyseal dysplasia (NSED) formerly known as "Namaqualand hip dysplasia" [Beighton et al., 1984]. The two conditions are similar in that they are characterised by early-onset SED with OA, which progresses and leads to physical handicap by adulthood [Learmonth and Beighton, 1987; Sher et al., 1991]. Furthermore, both conditions are inherited as autosomal dominant traits and have been linked to the COL2A1 gene [Sher et al., 1991; Ramesar and Beighton, 1992]. In the present investigation, the role of COL2A1 in the pathogenesis of the SEDCT and NSED phenotypes was examined. It was

shown that the two conditions share a common haplotype thus implying a founder effect. DNA sequencing studies revealed a common mutation within the COL2A1 gene for both disorders.

4.2. METHODS AND MATERIALS

(i) The Family:

SEDCT

The SEDCT phenotype is characterised by stunted stature with predominant truncal shortening (Fig. 4-1). Moderately severe changes in the hip joint and vertebral bodies are evident and there are no extraskeletal manifestations. The propositus in the affected kindred had initially been diagnosed as having "dwarfism with premature degenerative OA of the hips" and his affected children were misdiagnosed as having "bilateral Perthes disease" [Beighton, personal communication]. The condition usually has its onset at puberty although a symptomatic six-year old child has been encountered. Progressive discomfort and disability can be alleviated by prosthetic hip replacement. Spinal malalignment was not observed in affected individuals but backache was common in adulthood.

Radiographic changes are generally confined to the hip joint and spine, although involvement of the upper tibial epiphyses was present in two affected individuals. The original molecular investigation of type II collagen involvement in SEDCT was undertaken in the 3-generation family comprising 12 affected individuals. Southern blot analysis using COL2A1 probes (HcolIIc [Sangiorgio et al., 1985] and HcolIIFB1

Table 4-I. Pathogenic glycine substitutions in type II collagen.

SUBSTITUTION	EXON	CHONDRODYSTROPHY	REFERENCE
Gly 67 Asp	10	Wagner	Korkko et al., 1993
Gly 103 Asp	12	Kniest	Wilkin et al., 1994
Gly 154 Arg	15	SED	Vikkula et al., 1993a
Gly 247 Ser	19	mild SED	Ritvaniemi et al., 1994
Gly 274 Ser	21	mild SED	Winterpacht et al.,1994a
Gly 292 Val	21	SEMD	Tiller et al., 1995
Gly 304 Cys	21	SEMD	Tiller et al., 1995
Gly 574 Ser	33	hypochondrogenesis	Horton et al., 1992
Gly 604 Ala	35	hypochondrogenesis	Freisinger et al., 1994
Gly 691 Arg	38	achondrogenesis type II	Mortier et al., 1995
Gly 709 Cys	39	SEMD	Tiller et al., 1995
Gly 769 Ser	41	Achondrogenesis type II	Chan et al., 1995
Gly 805 Ser	41	hypochondrogenesis	Bonaventure et al.,1995
Gly 853 Glu	43	hypochondrogenesis	Bogaert et al., 1992
Gly 895 Ser	44	SED	Winterpacht et al.,1995
Gly 943 Ser	46	achondrogenesis type II	Vissing et al., 1989
Gly 997 Ser	48	SEDC	Chan et al., 1991

[Strom et al., 1988]) demonstrated co-segregation of restriction length polymorphisms (RFLPs) with the disorder [Ramesar and Beighton., 1992]. A two point LOD score of 4.51 at θ=0.00 was obtained between SEDCT and the COL2A1 markers (Table 4-II).

NSED

The NSED phenotype, so named because the affected persons have their antecedents in Namaqualand (a geographical region within the Northern Cape province of South Africa), is characterised by progressive degenerative OA predominantly in the hip joint and vertebral bodies. Affected individuals develop pain in the hips and in many instances, hip joint replacement surgery is necessary. NSED differs from SEDCT in that the condition has its onset in early childhood and the height of affected individuals is similar to their unaffected relatives (Fig. 4-2). Myopia which is present in some forms of SED, is not a consistent feature in NSED [Sher et al., 1990].

In NSED the femoral capital epiphyses are flattened and fragmented at an early age [Sher et al., 1990]. Prosthetic hip replacement is usually necessary as secondary OA develops in adulthood. Mild vertebral changes are present in about 60% of the affected individuals, with mild spinal malalignment and lumbar lordosis in a minority.

A molecular investigation in a 5-generation NSED family, comprising 12 affected individuals, yielded a maximum two point LOD score of 7.98 at θ=0.00 between the disorder and the COL2A1 markers [Sher et al., 1991] (Table 4-II).

Fig. 4.1. The SEDCT kindred. Affected individuals are indicated with arrows.

Fig. 4.2. The NSED kindred. Affected individuals are indicated with arrows.

Table 4-II. Two point LOD scores obtained for Southern blot/ RFLP analysis with COL2A1 RFLP-probes.

θ	0.00	0.05	0.10	0.20	0.30	0.40	REFERENCES
NSED	7.98	7.26	6.51	4.93	3.21	1.42	Sher et al., 1991
SEDCT	4.51	4.16	3.78	2.96	2.04	0.97	Ramesar and Beighton, 1992

(ii) Molecular Genetic Investigations

The primary aim of the present study was to examine the COL2A1 gene for mutations which were causing the SED phenotype in SEDCT and NSED. Thereafter, haplotypes were established using PCR-based microsatellite markers which were closely linked to the COL2A1 gene in order to determine whether the molecular defects in each kindred were genealogically related to each other.

Linkage Analysis

Prior to 1990, blood specimens had been collected, with informed consent, by standard venipuncture techniques. DNA was extracted using the Genomix^{TM} blood scale-up kit [*Talent*, Italy] (see Appendix 1, section 1.1.). Southern blot/RFLP studies had been performed [Sher et al., 1991; Ramesar and Beighton, 1992], and the remaining DNA stored for further analysis. The same DNA specimens were retrieved for investigation in the present study. The COL2A1 chromosomal region, 12q13.11 - 12q13.12 [Takahashi et al., 1990], was genotyped using primers for the COL2A1 3' VNTR [Stoker et al., 1985] and the microsatellite markers D12S361 and D12S368

[*Research Genetics*, USA]. The localisation of these markers on chromosome 12 is given in Ch.2, section 2.2.1. Following standard PAGE, autoradiography and allele scoring.

The PCR reactions were performed as described in Appendix 1 (section 2.1.2), with 100-200ng of genomic DNA as templates; 5-10pmol of each primer; 1μCi of α-[32P]dCTP; 200uM dATP, dCTP, dGTP and dTTP; and 0.5U of *Taq* polymerase, in a final volume of 10μl. Samples were cycled 25 times in an Omnigene PCR machine using an annealing temperatures of 55°C and the PCR products were resolved by PAGE and visualised by autoradiography (Appendix 1, section 2.1.3.).

Linkage analyses were performed using the LINKAGE package Version 5.03 [Lathrop and Lalouel, 1984] and the map order from the Généthon map of microsatellite markers for each chromosome. In all analyses the number of alleles for each marker was reduced to five, without loss of information in the family, in order to accommodate the computer program's limitations for numbers of alleles. Male and female recombination rates were taken to be equal. The condition was assumed to be autosomal dominant with full penetrance and a gene frequency of 0.001 was assumed for the disease locus.

Mutation Analysis

The exons correlating to the triple helical domain of COL2A1 and their flanking sequences were amplified by a series of intronic primers (see Ch. 3, Table 3-II) using genomic DNA from affected persons as a template. Specimens from unaffected

relatives and spouses were used as controls. The PCR products were screened for mutations using SSCP analysis and confirmed by conformation sensitive gel electrophoresis (CSGE) by Dr Jarmo Korkko (as decribed in Appendix 1, section 2.2.4) of the Allegheny University of the Health Sciences, Philadelphia, USA.

Exons in which conformation polymorphisms were observed, were sequenced as described in Appendix 1 (section 2.2.2). The double-stranded PCR products were purified using the QIAquick protocol for eluting DNA from a gel matrix and sequenced, in both 5' and 3' directions, using the Sequenase version 2.0 DNA sequencing kit. Sequencing reactions were analysed on 6% PAGE gels which were dried at 80^{o}C and visualised by autoradiography.

4.3 RESULTS

The molecular study involved the confirmation of linkage of the SEDCT and NSED phenotypes to COL2A1 using closely linked microsatellite markers for PCR studies. The pedigrees of the portions of the kindreds which were used for linkage analyses are shown in Fig. 4.3 and Fig. 4.4. No recombinations were observed using the intragenic VNTR and D12S368, D12S361 and D12S339. The LOD scores for SEDCT and NSED are listed in Tables 4-II and 4-III, respectively. Using these microsatellite markers the phase of the disorder and the associated haplotype was established. A common haplotype was found to be cosegregating with the two phenotypes (Fig. 4.3 and Fig. 4.4, respectively). Thereafter SSCP/CSGE analysis and DNA sequencing were performed in order to identify the determinant mutation in each disorder.

Using the intronic primers for SSCP, a conformational polymorphism was detected in exon 29 of the triple helical domain of COL2A1 and was confirmed by CSGE analysis. The variant SSCP pattern cosegregated with both the SEDCT and NSED phenotypes (Fig. 4-3 and 4-4, respectively). Neither the unaffected family members nor their spouses exhibited the SSCP. This conformational change, upon sequencing, was found to be due to a G21011T transversion (Fig. 4-5) which resulted in a Gly472Cys substitution in the type II collagen molecule.

4.4 DISCUSSION

In this study, a common haplotype and, subsequently, a common COL2A1 mutation, has been identified in two families having two slightly different forms of SED. The two phenotypes, SEDCT and NSED, had previously been linked to COL2A1 using RFLP analyses [Sher et al., 1991; Ramesar and Beighton, 1992]. In the present study, linkage analysis confirmed that the two autosomal dominant traits cosegregated with DNA polymorphic markers from the COL2A1 locus. Furthermore, the haplotype which was associating with SEDCT was found to be the same as for NSED.

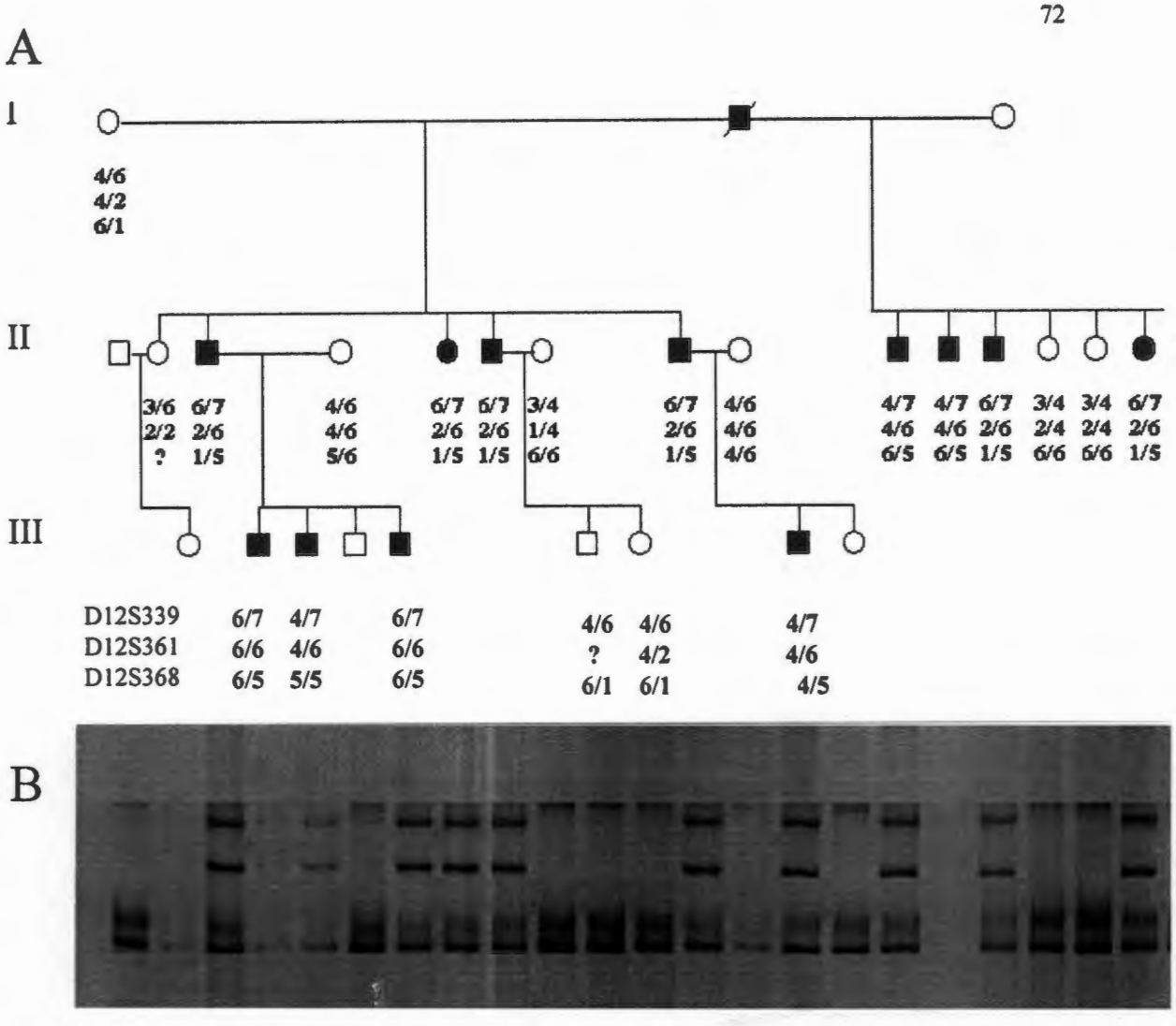

Fig. 4.3. Haplotypes (A) and PCR-SSCP (B) analysis of exon 29 of the COL2A1 gene in SEDCT. The SSCP change cosegregates with the disorder in the family. Haplotypes are indicated below each symbol. Shaded symbols represent the affected individuals and unshaded symbols the unaffected individuals.

Table 4-III. Two point LOD scores obtained for microsatellite analysis with COL2A1 polymorphic markers in the SEDCT family. The genetic distances between the markers and their proximity to the gene is given in Ch.2, Fig. 2-1.

SEDCT	0.00	0.05	0.10	0.20	0.30	0.40	0.45
D12S361	4.82	4.44	4.03	3.17	2.18	1.05	0.44
D12S368	4.52	4.14	3.74	2.89	1.20	0.91	0.38
VNTR	4.82	4.44	4.04	3.17	2.18	1.05	0.45

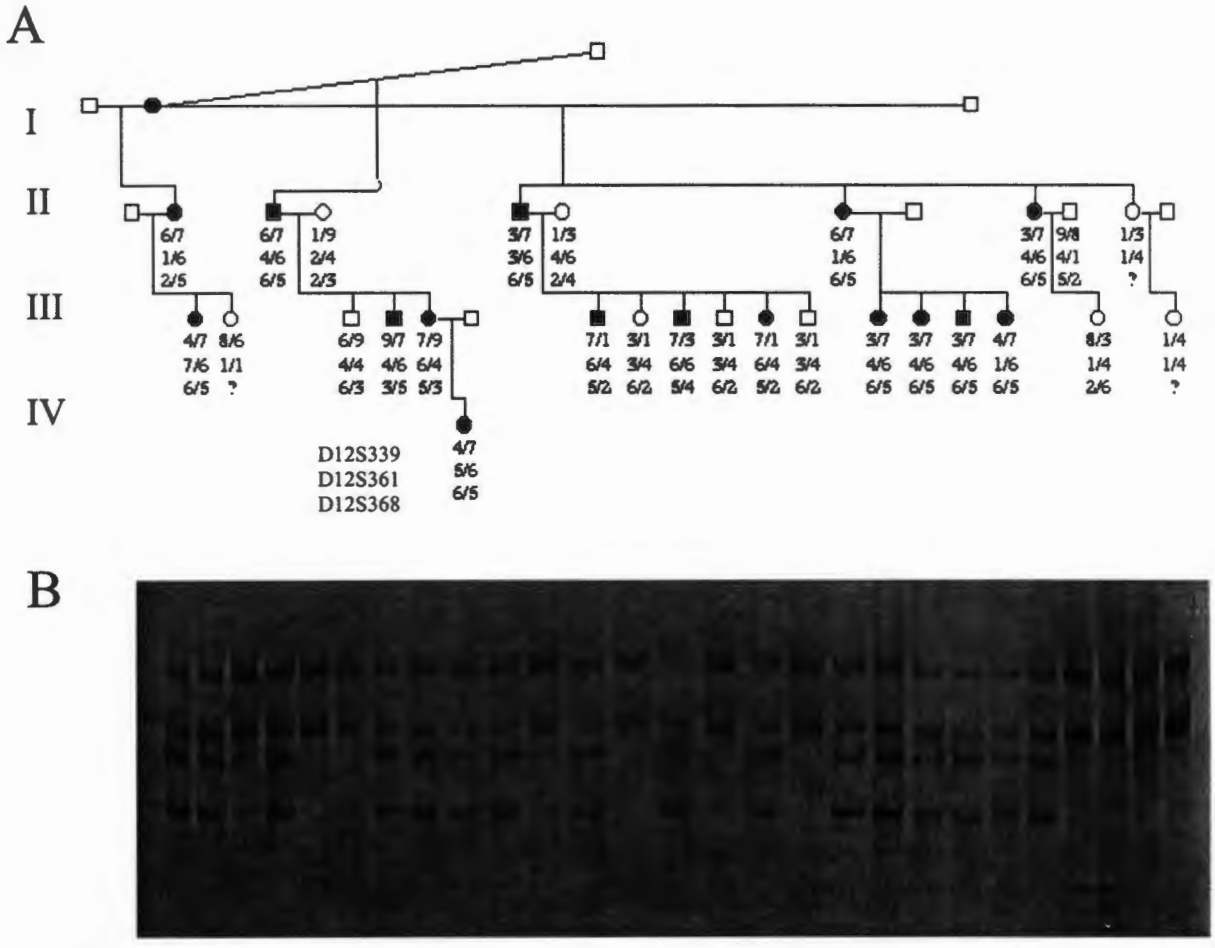

Fig. 4-4. Haplotypes and PCR-SSCP analysis of exon 29 of the COL2A1 gene in NSED family. Shaded symbols represent the affected individuals and unshaded symbols are the unaffected individuals. A.Haplotypes are indicated below each symbol. B.The SSCP change cosegregates with the disorder in the family.

Table 4-IV. Two point LOD scores obtained for microsatellite analysis with COL2A1 polymorphic markers in the NSED family. The genetic distances between the markers and their proximity to the gene is given in Ch.2, Fig. 2-1.

SED	0.00	0.05	0.10	0.20	0.30	0.40	0.45
D12S361	6.02	5.53	5.01	3.89	2.62	1.18	0.44
D12S368	4.01	3.52	3.37	2.70	1.78	0.70	0.21
VNTR	4.12	3.72	3.32	2.46	1.56	0.64	0.21

(a).

nt position 21011

CCT GGC CCT CCT **GGT** CCC CCA normal sequence

Pro Gly Pro Pro **Gly** Pro Pro

aa position 472

CCT GGC CCT CCT **T**GT CCC CCA variant sequence

Pro Gly Pro Pro **Cys** Pro Pro

(b).

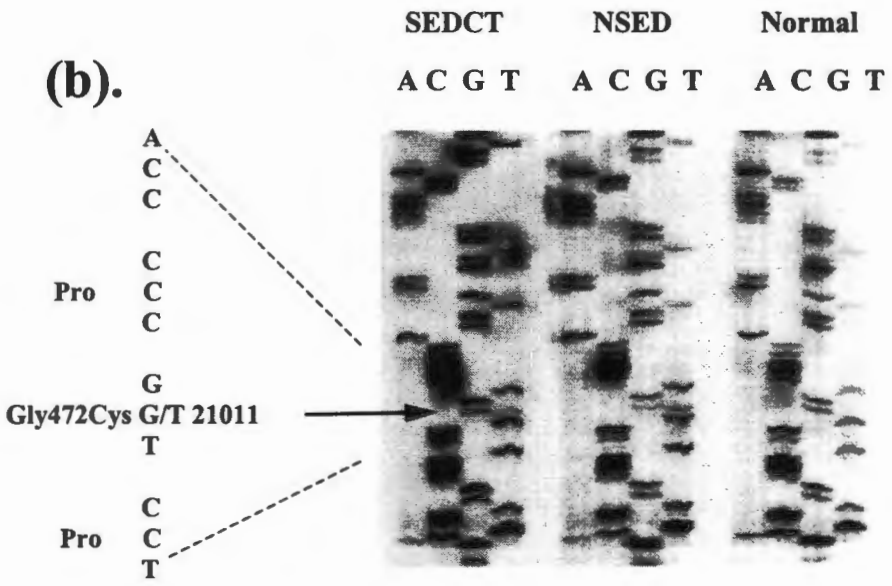

Fig. 4-5. Results of sequence analysis of COL2A1 exon 29 in SEDCT and NSED (a) Partial sequence of exon 29. The normal and abnormal alleles are shown. (b) The sequencing gel showing the region of exon 29 in which the G21011T transversion occurs. The arrow indicates the altered nucleotide.

In an extensive search for the mutation using CSGE analysis, a conformational change was observed in exon 29 of COL2A1. The cause of the condition in each family can probably be attributed to the Gly472Cys substitution which results from this mutation. Although the base change, which is common to SEDCT and NSED, has not been reported before, the substitution of a glycine residue by cysteine, which is not normally found in the triple helix of type II collagen, has previously been implicated in the aetiology of chondrodysplasias (Table 4-V). Garofalo et al. [1991] showed that a Gly85Cys substitution in transgenic mice produced a lethal chondrodysplasia which was characterised by short limbs and trunk, craniofacial abnormalities and cleft palate. The histological features and the cartilage ultrastructure resembled that of the human SEDC phenotype. In humans, Gly304Cys and Gly709Cys [Tiller et al., 1995] substitutions have been found to cause SEMD which has some features of SEDC. In some instances of SEMD severely stunted stature, pectus carinatum and scoliosis are a feature [Murdoch and Walker, 1969]. In keeping with the phenotypic spectrum of severity in the collagen triple helix, Gly/Cys substitutions close to the carboxyl terminus reportedly cause the lethal condition, hypochondrogenesis, which is a form of short-limbed dwarfism [Mortier et al., 1995].

The phenotypes of SEDCT and NSED are both milder than the chondrodysplasias caused by the other Gly/Cys substitutions. Stunted stature was, however, an (albeit inconsistent) feature of the SEDCT phenotype. Furthermore, although there was no observed spinal malalignment, backache was common in the affected persons in this family. In the NSED family, the affected persons were not markedly shorter than their unaffected siblings. Mild spinal malalignment was observed in four people. There was no metaphyseal involvement in either of the kindred.

Table 4-V. Chondrodysplasia-causing gly/cys substitutions in type II collagen.

MUTATION	CHONDRODYSPLASIA	REFERENCE
Gly304Cys	SEMD	Tiller et al., 1995
Gly472Cys	SEDCT/NSED	Present study
Gly709Cys	SEMD	Tiller et al., 1995
Gly910Cys	Hypochondrogenesis	Mortier et al., 1995
Gly943Cys	Hypochondrogenesis	Mortier et al., 1995

The reported SED-causing COL2A1 mutations have all been heterozygous mutations which map to the triple helical domain of the type II collagen molecule (Table 4-I), and are thought to act in a dominant-negative manner at the molecular level [Horton et al., 1995]. The most common SED-causing mutations appear to be Gly/Ser substitutions. The Gly residues are considered to be critical for proper assembly of collagen chains into triple helices which occurs from the carboxy propeptide in the direction of the amino terminus. The substitution of Gly disturbs helix formation with adverse functional consequences. Collagen molecules which contain the mutant chains are thought to undergo premature degradation resulting in haploinsufficiency or they incorporate the mutant strands into collagen fibrils and affect the properties and function of these fibrils. In the South African family a Gly/Cys substitution results in a milder phenotype than reported for other Gly/Cys changes (Table 4-V). It has been proposed that the local amino acid context in which the amino acid substitution occurs may also contribute to variations in the phenotypic gradient of expression [Bąchinger et al., 1993]. It may therefore be prudent to suggest that in the South African family, the Pro residues which flank Gly472, protect the collagen monomer to some degree from the effect of the Cys substitution. Since, none of the other Gly residues which have been substituted by Cys (Table 4-V) have a similar amino acid environment, the resultant phenotypes are predictably severe.

The question arises whether SEDCT and NSED represent two separate, unrelated kindreds or whether they share a common progenitor in whom the mutation originated. The latter situation would be in keeping with the fact that both kindreds belong to the mixed ancestry population of South Africa, who have European, Khoi, Malay, and San admixture. The molecular findings, common haplotype and common genetic defect, suggest that SEDCT and NSED represent the same SED entity with mild variation in the final clinical manifestations. The phenomenon of variable phenotypic expression resulting from a single gene defect has previously been reported for the collagen genes and skeletal disorders. A mutation in exon 11 of COL2A1 which causes an Arg75Cys substitution results in two different phenotypes, one with the typical stunted stature of SED [Williams et al., 1993] and the other with a tall stature [Bleasel et al., 1995]. Those authors suggested that the height difference reflects the complex multigenic factors which determine height. Furthermore, they proposed that the variable phenotypes caused by the single gene defect highlights the complex interrelationships between the underlying molecular events and the final clinical expression of the disorder. In another instance, Byers et al. [1991] proposed that the phenotypic variability which was observed in osteogenesis imperfecta as a result of mutations in the COL1A1 gene, was due to many contributing factors including the type of mutation, the position of the mutation, the nucleotide environment as well as epigenetic factors.

The best explanation for the identity of the genotype and the similar phenotype of SEDCT and NSED seems to be that they harbour the same founder mutation. The haplotype analyses, linkage, and ethnic background provide evidence of a genealogical link between the two families and the same ancestral mutation. It is likely that the variable clinical expressivity between the two skeletal disorders is due to the influence of additional genetic or environmental factors.

CHAPTER 5:
MULTIPLE EPIPHYSEAL DYSPLASIA (MED)

5.1 INTRODUCTORY BACKGROUND

Multiple epiphyseal dysplasia (MED) is an autosomal dominant disorder which is characterised by abnormalities of the epiphyseal growthplates of tubular bones. Minor involvement of the metaphyses and vertebrae is sometimes present [Beighton, 1988]. In affected persons, abnormal articular surfaces lead to degenerative joint disease particularly of the hip and knee joints [Hulvey and Keats, 1969].

There is a spectrum of severity within MED; the severe Fairbank type [Fairbank, 1946] is characterised by short, stubby fingers and short limbs and usually presents in mid childhood. The milder Ribbing type presents as precocious OA of the hip with mild short stature and minimal involvement of the hands [Ribbing, 1937]. There is, however, considerable overlap between the phenotypes with both inter- and intra-familial variation. Radiographic features of MED are age-related, and the changes in the epiphyses, digits and, in some instances, the spine, may be more evident with advancing age.

Although the classical forms of MED, SED and pseudoachondroplasia (PSACH) are clinically and radiographically distinct, there can be some overlap within the phenotypic spectrum [Beighton, 1988]. Ultrastructural changes in cartilage from individuals with PSACH and individuals affected with MED, Fairbank type, provided evidence that these two conditions belonged to the same family of bone dysplasias [Stanescu et al., 1993]. Langer et al. [1993] also suggested that the two conditions were closely related. Molecular findings have subsequently demonstrated that some forms of MED are allelic with PSACH (*vide infra*).

Genetic heterogeneity in MED has been demonstrated by linkage to more than one genetic locus. The EDM1 locus on 19p13.1 [Oehlmann et al., 1994; Knowlton et al., 1995] encodes the cartilage oligomeric matrix protein (COMP) [Newton et al., 1994; Briggs et al., 1995]. The COMP gene has been identified, characterised and localized to the critical interval for EDM1 [Newton et al., 1994; Briggs et al., 1995]. COMP is a 524 kD pentameric glycoprotein of the cartilage extracellular matrix and is a member of the thrombospondin family of genes [Bornstein et al., 1993]. The protein localizes specifically to the territorial membrane of cartilage chondrocytes.

Mutations in the COMP gene result in either MED Fairbank or PSACH [Briggs et al., 1995; Hecht et al., 1995; Briggs et al., 1998]. In both disorders the mutations (Table 5-I) predominantly occur within the calmodulin-like repeat domain of the protein which encodes a calcium-binding motif. Aspartic acid residues which line this domain are positioned so that they can bind calcium ions. The localisation of MED- and PSACH-causing mutations in this domain, suggests that COMP plays

Table 5-I. A summary of published MED phenotypes and their determinant mutations.

REFERENCE	MED TYPE	AGE OF ONSET	JOINT PAIN	STATURE	HANDS	GENE	MUTATION	REGION OF PROTEIN
Weaver et al, 1993	hip OA	<12yrs	hips & knees	mildly short	stubby digits	COMP	not defined	not defined
Deere et al., 1995	Fairbanks	<5yr	hips	stocky	10-75th percentile	COMP	not defined	not defined
Briggs et al., 1995	Fairbanks	———	——	———	———	COMP	exon asp342tyr	3rd calmodulin repeat
Ballo et al., 1997	Ribbing	<5 yrs	hip & other	short	short digits	COMP	exon 14 asn523lys	adjacent to calmodulin rpt
Susic et al., 1997	Fairbanks	———	——	———	———	COMP	exon cys371ser	4th calmodulin repeat
Briggs et al., 1998	Fairbanks	<15 yrs	hips & knees	short	normal	COMP	exon 13 asn453ser	7th calmodulin repeat
Briggs et al., 1998	MED/ PSACH?	childhood	——	short	all segments short	COMP	exon 16 thr585arg	COOH
Briggs et al., 1994 Muragaki et al.,1996	Fairbanks	2,5-6yr	knees & ankles	mildly short	stubby digits	COL9A2	exon 3 splice mutation	N-term of 3rd collagenous domain
Deere et al., 1995	Fairbanks	early adult	all joints	short	prominent joints	unlinked	——	———

an important role in calcium-binding within the cartilage extracellular matrix. More recently, Briggs et al. [1998] reported on mutations in the carboxy-terminal domain in PSACH and MED. Their findings imply an important structural or functional role for this domain in the cartilage extracellular matrix.

The COMP mutations which have been identified in individuals with MED result in the substitution of a conserved aspartic acid residue at position 342 by a tyrosine residue in the third calmodulin-like repeat [Briggs et al., 1995] and a Cys371 Ser substitution in the fourth calmodulin-like repeat [Susic et al., 1997]. Both mutations result in the severe MED, Fairbank type, phenotype. Using SSCP and DNA sequencing, Hecht et al. [1995] identified eight COMP mutations in persons with PSACH. In six of these individuals the mutation was a deletion or substitution of a conserved aspartic acid residue which lined the calcium-binding domain. Another PSACH-causing mutation involved a 3 bp deletion which resulted in the elimination of a serine residue at position 459 in this domain. Briggs et al. [1995] identified a Cys328Arg substitution in the second calmodulin-like repeat of COMP in a family with moderately severe PSACH and a deletion of a conserved aspartic acid residue between positions 372 and 374, in the fourth calmodulin-like repeat in another family with PSACH of similar severity. Susic et al. [1997] also reported a 12bp deletion in the calcium binding domain which resulted in a mild PSACH phenotype. Briggs et al. [1998] reported on a further 12 mutations in this domain, and two others which occurred in the carboxy-terminus: Thr585Met in a person with mild PSACH, and Thr585Arg in a person with MED.

The EDM2 locus on 1p32 which encodes COL9A2 [Perälä et al., 1993; Warman et al., 1994] was shown to be linked [Briggs et al., 1994] in a kindred which had

characteristic MED features, but with only mild hip involvement. In this family a splice site mutation caused exon skipping and an in-frame deletion of 12 amino acid residues [Muragaki et al., 1996]. The resultant phenotype was mild, and the digits were not affected. Those authors suggested that mutations in COL9A2 apparently have a stronger effect in the knees than do COMP mutations. This hypothesis is supported by the observation that the MED family which has been linked to COL9A2 has only knee pain with no clinical evidence of hip involvement, whereas the persons with MED linked to COMP, complained of hip and knee joint pains and, in some instances, discomfort was also experienced in other joints (Table 5-I). The symptoms of articular pain does not, however, necessarily correlate with the extent of involvement of the joint, and the value of using the anatomical location as an indicator of the underlying defect remains questionable. Additional genetic heterogeneity in MED has been demonstrated in two families; one with three affected siblings in a family of seven children in which neither of the parents was affected. The other family had classical features of AD-MED, type Fairbank. A number of candidate collagen genes which were tested for linkage to the condition in this family were excluded under models for dominance and recessiveness [Deere et al., 1995; Briggs and Cohn, unpublished data].

In the course of the current study, a South African family of western European stock with a mild form of MED was investigated. Linkage analysis with a number of candidate loci was undertaken in order to identify a candidate gene. The mutant gene, COMP, was localised and the pathogenic mutation was identified during the course of screening exons 10-14 (the region in which all of the

published mutations had been found) of that gene. The molecular findings which will form the subject of this chapter have been published during the course of this project [Ballo et al., 1997].

5.2. METHODS AND MATERIALS

(i) The Family

A South African family of western European stock was investigated. The affected family members, the proposita, her son, her brother and her deceased mother were shorter in height (154cm, 156cm, 157cm and 152cm) than their unaffected relatives who were of normal stature. Joint pains, mainly in the hips, but also in other large joints, were the predominant clinical problem. There was some limitation in the range of movement of the affected joints, but articular mobility was otherwise unremarkable. Three affected persons had received bilateral prosthetic hip replacements in adulthood. All the affected relatives had shortened digits.

Radiographs of the proposita and her affected son showed significant flattening and irregularity of femoral heads, with some joint space narrowing and periarticular sclerosis. Other large joints were similarly affected, indicating secondary generalised OA (Fig. 5-1). The end plates of the vertebral bodies showed mild sclerosis and irregularity but there was no significant flattening. The skeletons were otherwise radiologically normal and in particular, there were no changes in the diaphyses or metaphyses of the tubular bones. (For this reason the diagnosis of PSACH, which is allelic to MED, could be discounted).

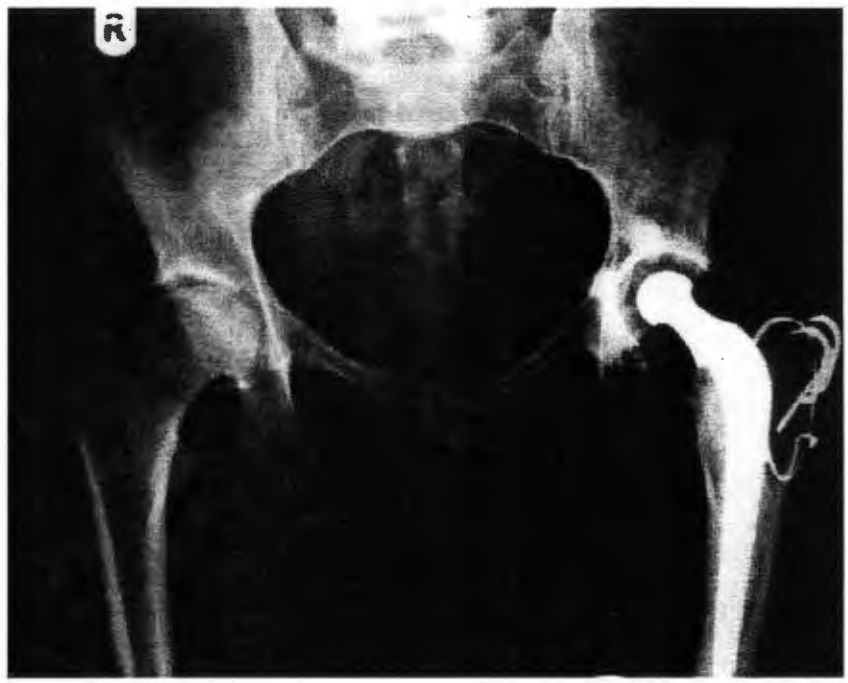

Fig. 5-1. MED kindred: Antero-posterior radiograph of the pelvis of the proposita at the age of 45 years. The femoral head on the right side is irregular and somewhat flattened. Areas of patchy sclerosis and lucency are evident. Prosthetic joint replacement is evident on the left side.

In the hands, the phalanges were mildly short but not otherwise dysplastic (Fig. 5-2a). Pattern profile analysis, using the method of Poznanski et al. [1972], showed that these changes were maximal in the metacarpals, with a trend to lesser severity in the distal phalanges (Fig. 5-2b).

(ii) Molecular Genetic Investigations

Linkage Analysis

Twenty ml of venous blood samples were collected under sterile conditions from each of 7 family members (2 affected and 5 unaffected) into tubes containing the anticoagulant, EDTA. The DNA was prepared by using the Genomix blood scale-up kit [*Talent,* Italy] (Appendix 1, section 1.1). In order to localise the disease gene, microsatellite markers which are in close physical proximity to the candidate genes, COL9A1 and COL9A2 (see Ch. 2, section 2.2.3), and COMP (see Ch. 2, section 2.5.3.), were chosen. The microsatellite markers were typed by PCR, and the alleles resolved by PAGE (Appendix 1, section 2.1.3.).

The PCR reactions were performed as described in Appendix 1 (section 2.1.2) with 100-200ng of genomic DNA as templates; 5-10pmol of each primer; 1μCi of α-[32P]dCTP; 200uM dATP, dCTP, dGTP and dTTP; and 0.5U of *Taq* polymerase, in a final volume of 10μl. Samples were cycled 25 times in a Omnigene PCR machine and the PCR products were resolved by PAGE and visualised by autoradiography.

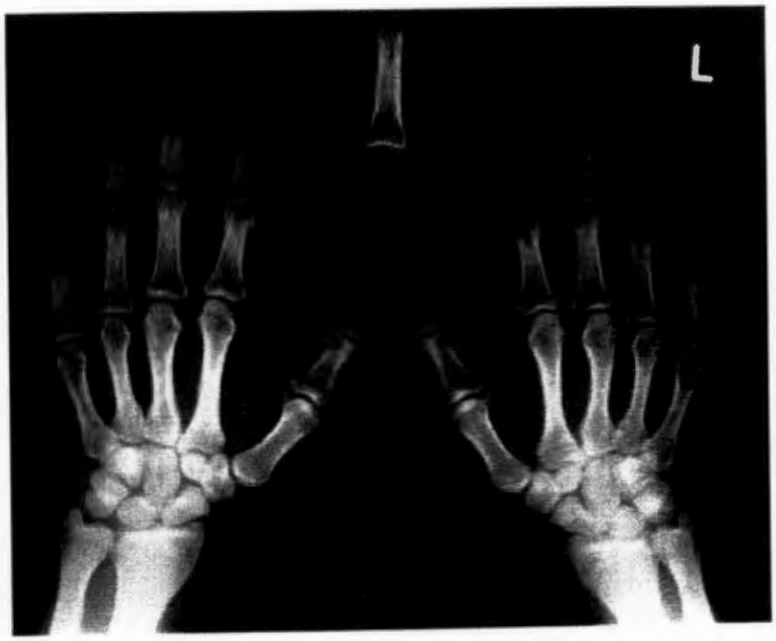

Fig. 5-2(a). MED kindred: Antero-posterior radiographs of the hands of the proposita's son at the age of 21 years. The tubular bones are shortened but not dysplastic.

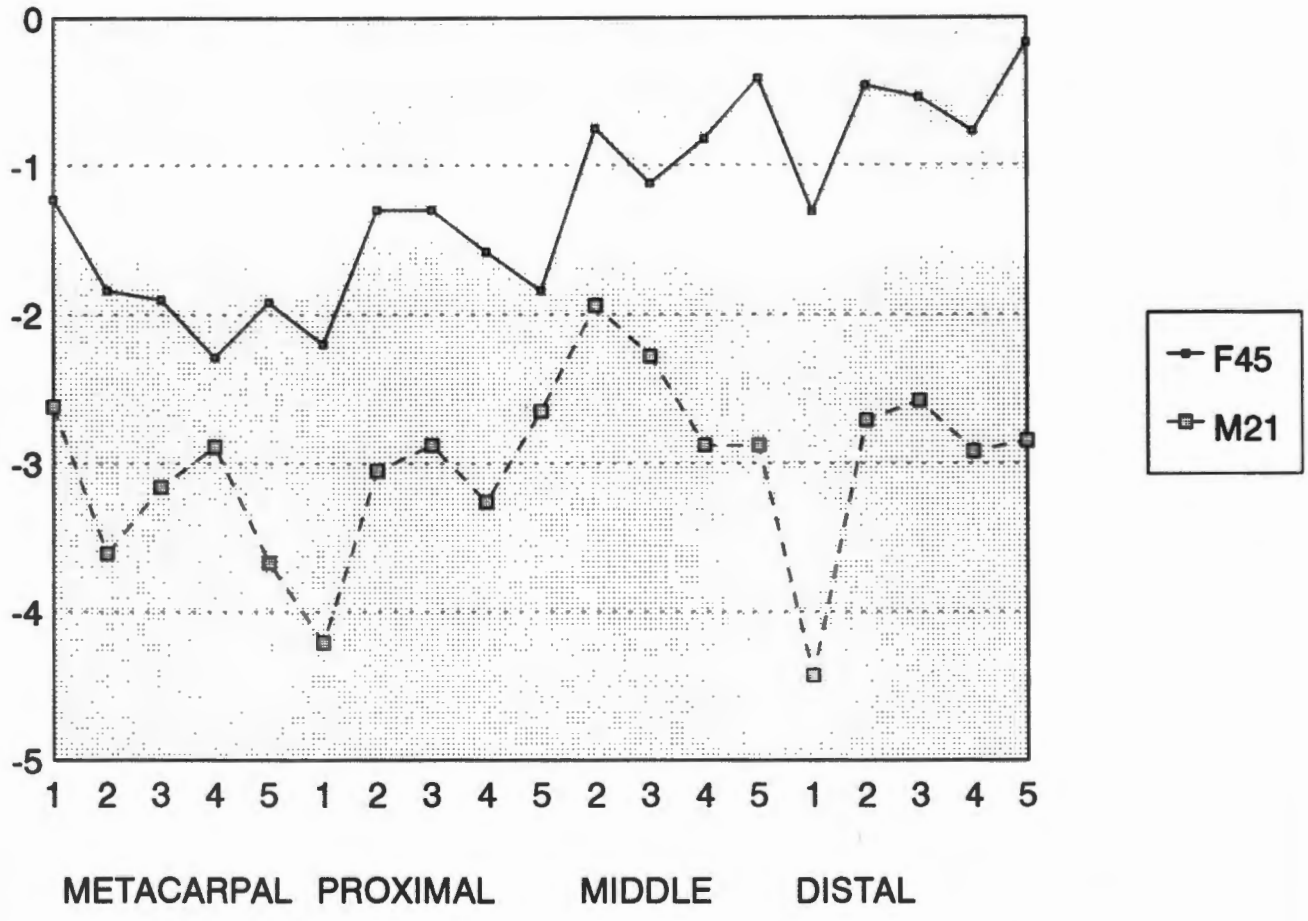

Fig. 5-2(b). MED kindred: Pattern profile analysis of the digits of the proposita age 45 (F45) and her son, age 21 (M21). Both have shortness of all tubular bones with a minor trend towards decrease in severity from the metacarpals to the distal phalanges.

Linkage analyses were performed using the LINKAGE package Version 5.03 [Lathrop and Lalouel, 1984] and the map order from the Généthon map of microsatellite markers for each chromosome. In all analyses the number of alleles for each marker was reduced to five, without loss of information in the family, in order to accommodate the computer program's limitations for numbers of alleles. Male and female recombination rates were taken to be equal. The condition was assumed to be autosomal dominant with full penetrance and a gene frequency of 0.001 was assumed for the disease locus.

Mutation Analysis

Candidate genes were further assessed for disease-causing potential by mutation screening through SSCP analysis. Following the observed lack of recombination between the MED phenotype and the COMP gene, exons 10-14 and flanking sequences were amplified using a series of intronic primers (Table 5-II) (exons 10-14 of the COMP gene were the first exons to be screened since this was the region within which most of the MED/PSACH mutations had reportedly been found). The PCR was performed in a total volume of 25µl using primer-specific annealing temperatures (Table 5-II). The DNA strands were resolved on MDE gels and the SSCPs visualised by silver staining as described in Appendix 1 (section 2.2.1.2).

Following the observation of SSCPs, the relevant exon was sequenced in order to determine the nucleotide changes which resulted in the bandshifts. The SSCP bands were excised from the PAGE gel. The single stranded DNA was eluted in sterile distilled water, purified using the QIAquick protocol and sequenced, using the Sequenase version 2.0 DNA sequencing kit (see Appendix, section 2.2.2) as recommended by the manufacturer [USB, USA]. Sequencing reactions were analysed on 6% PAGE gels and visualised by autoradiography.

Table 5-II. Oligonucleotide primers for PCR amplification of exons 10-14 of the COMP gene (as described in Briggs et al., 1998).

Exon	PCR Primer	Product size (bp)	Annealing Temperature (°C)
10	5'-TGAGGAGTGTGACCTTTGCC-3' 5'-AGCCGAATCCCGCCTTCGGTG-3'	279	55
11	5'-CTTGGGCTCTGGTCCCGTGG-3' 5'-GCTTACCCAGCTGGAGTCTG-3'	181	57
12	5'-ATTTCCTCTGTCTGATTATGG-3' 5'-CCAGAGACAATGAGCTCTCCAG-3'	168	57
13	5'-GGGTAGCCTTTGACAAAACG-3' 5'-GTTAGGCACCAGGCGGCAG-3'	223	55
14	5'-TGACTTTAGCCCACCGAGGG-3' 5'-CTCAGCATAGGCCTCACTGTG-3'	281	57

NOTE- For each exon the forward or sense primer is given on the top line and the reverse (antisense) primer is given below. DNA was amplified under the following PCR conditions: denaturation at 94°C for 30 secs, annealing temperature as tabulated above and extension at 72°C for 30 secs.

5.3. RESULTS

The structure of the family under investigation is shown in the pedigree in figure 5-3(a). Linkage analysis which is expressed as LOD scores, is presented in Table 5-III. A LOD score of -5.52 at θ=0.00, led to the exclusion of COL9A2. No recombinations were observed between the condition and the markers associated with the COL9A1 and COMP loci. The highest observed LOD scores were 0.78 (D19S602 at EDM1) and 0.81 (509-8B2 and 509-12B1 at COL9A1) at θ=0.00.

SSCP analysis of exons 10-14 of COMP revealed a bandshift in an amplified fragment containing exon 14. This polymorphism cosegregated with the condition in the family without exception (Fig. 5-3b) and was not seen in either the unaffected relatives or 50 random control samples representing unaffected individuals from the South Africans of western European origin.

DNA sequencing of exon 14 of COMP revealed a C to G transversion at nucleotide position 1594 (Fig. 5-4) in affected individuals and implied substitution of an uncharged polar asparagine residue by a charged lysine residue at position 523 of the COMP protein. Because of this significant finding at the EDM1 locus, COL9A1 which showed no recombination with the MED phenotype was not investigated further.

Table 5-III. Two point linkage analyses between MED in the South African kindred and polymorphic microsatellite markers from the candidate gene loci.

GENE	[CA]n marker	0.00	0.05	0.1	0.2	0.3	0.4
COL9A1	509-12B1 509-8 B2	0.75 0.81	0.66 0.71	0.56 0.61	0.37 0.40	0.19 0.21	0.05 0.06
COL9A2	MYCL1	-5.52	-0.52	-0.28	-0.09	-0.03	-0.005
COMP	D19S602	0.78	0.66	0.55	0.35	0.17	0.04

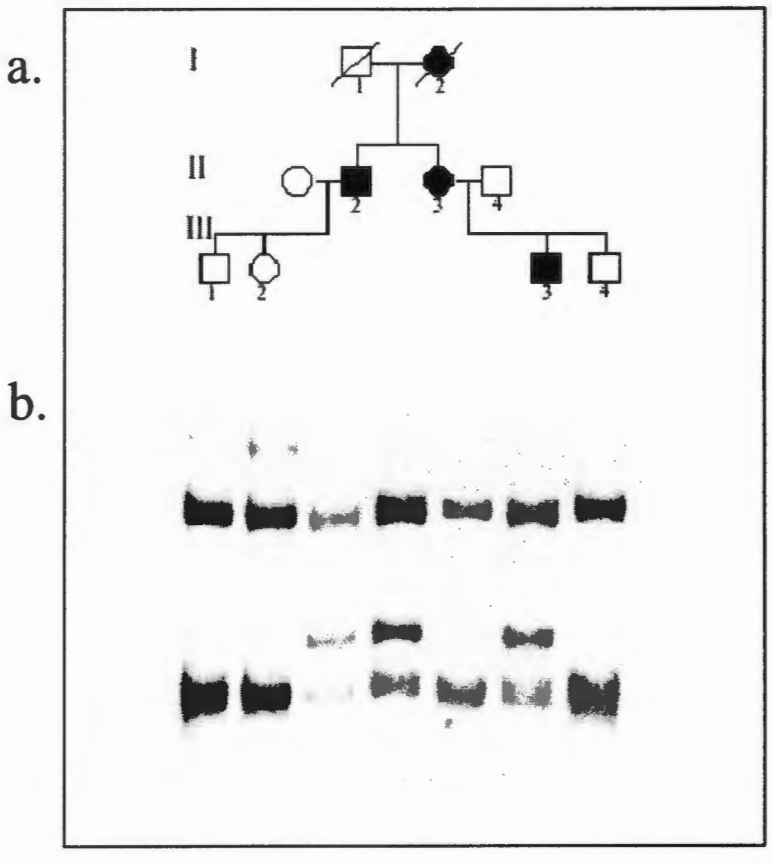

Fig. 5-3. MED kindred: Pedigree (a) and PCR-SSCP analysis (b) of exon 14 of the COMP gene in a South African MED kindred. Shaded symbols on the pedigree represent affected individuals. The SSCP change cosegregates with the disorder in the family.

a.

nt position 1594

TGT CCG AAG AAC GCT GAA GTC normal sequence

Cys Pro Glu **Asn** Ala Glu Val

aa position 523

TGT CCG AAG AA**G** GCT GAA GTC variant sequence

Cys Pro Glu **Lys** Ala Glu Val

b.

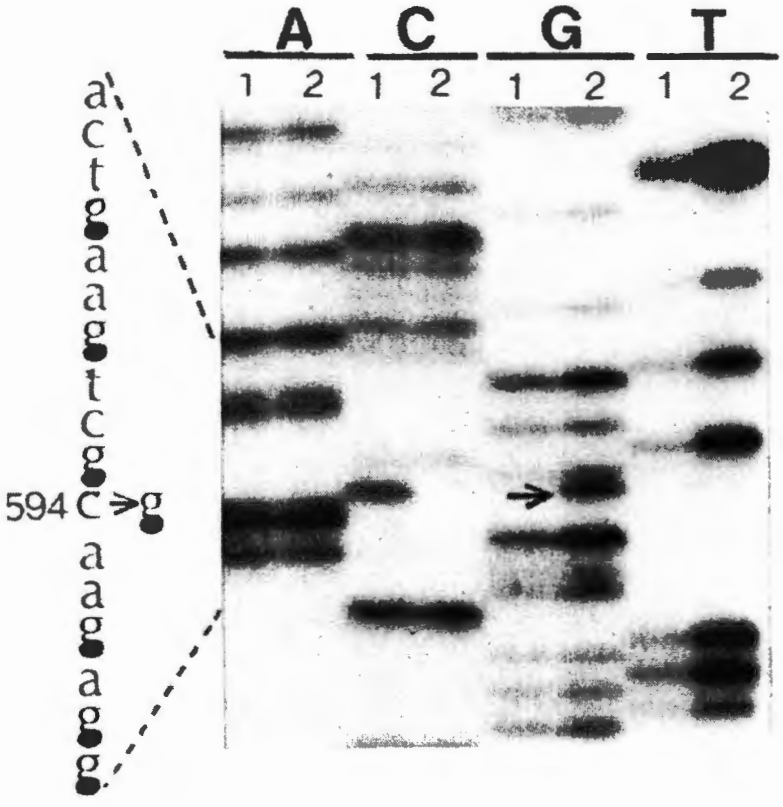

Fig. 5-4. MED kindred: (a). Partial sequence of COMP exon 14. The normal and abnormal alleles are shown. (b). Results of sequence analysis of single stranded DNA. The sequencing gel showing the region of exon 14 of the COMP gene in which the base substitution occurs. The arrow indicates the C-G transversion at nucleotide (nt) position 1594.

5.4. DISCUSSION

A mutation in the COMP gene has been identified in a South African family with a mild Ribbing type of MED. All of the COMP mutations which have been described elsewhere have resulted either in the severe, Fairbank, form of MED or PSACH. The clinical and radiographic features of these two phenotypes are distinct and diagnostic differentiation between the disorders is not difficult. It is becoming clear, however, that mutations in COMP unify phenotypes that span a phenotypic spectrum and, between the extremes, the phenotypic distinction may not be entirely clear cut; for instance, some patients with MED have mild changes in the metaphyses and vertebrae which could be regarded as a continuum with PSACH.

COMP mutations are thought to act through a dominant negative mechanism. If both normal and mutant COMP monomers are combined into pentameric structures, then only one out of 32 possible pentamers will comprise five normal monomers. Although the fate of mutant monomers and the effect of varying the proportion of mutant monomers in the pentameric molecule is not known, it is obvious that a disturbance of calcium binding is involved in the pathogenesis of the resultant chondrodystrophies. COMP mutations which result in the MED phenotype result primarily in the substitution of conserved aspartic acid and asparagine residues within the calmodulin-like domain which are thought to line the calcium-binding pockets and to bind calcium by charge-charge interactions. These mutations are thought to decrease the calcium-binding ability of the pockets, without producing major effects on the structure of this region of the molecule [Briggs et al., 1998]. In contrast, the changes causing PSACH has a major effect on the overall structure of this COMP domain in that it affects the relative positioning of the calcium-binding pockets.

Briggs et al. [1995] demonstrated that mutations in COMP, in the region that encodes a putative Ca++ binding motif, were responsible for MED type Fairbanks and PSACH. In addition, Hecht et al. [1995] independently identified mutations in COMP which were causative of PSACH. In the South African kindred, the Asn523Lys substitution, while not in an evolutionarily conserved position, is located immediately adjacent to a positionally conserved aspartic acid residue within the eighth calmodulin-like repeat of the molecule. If the asparagine at position 523 is also involved in the co-ordination of calcium ions, then a change in the local ionic environment, by the substitution of a larger, charged lysine residue, might reduce the charge-charge interactions which usually occur between Ca++ ions and the receptive amino acids in this region. As a consequence, the structural integrity of the COMP monomer may be compromised and/or an effect on calcium-dependent binding between COMP and other components of the extracellular matrix may result.

Due to the informativeness of the markers and the small family size, it is not surprising that an inheritance pattern which suggests an association to more than one locus, was observed. This finding highlights the potential uncertainty in using just linkage analysis in diagnostic studies for small families with a genetically heterogeneous disorder and argues that the identification of a mutation is necessary to confirm the association between the gene and the condition.

The identification of a mutation in the COMP gene in this family expands the range of phenotypes that can be produced by COMP mutations, defining a spectrum of allelic disorders from severe PSACH through mild MED.

CHAPTER 6:

FAMILIAL PRIMARY GENERALISED OSTEOARTHROPATHY

6.1. INTRODUCTORY BACKGROUND

Primary generalised OA is a common, slowly progressive disorder affecting mainly the weight-bearing joints and is a major cause of pain and disability in the elderly [Mankin et al., 1974]. Although the condition is mostly sporadic, familial clustering may be explained by shared genetic and environmental influences or a combination of the two [Wordsworth, 1995]. Twin studies have provided evidence for a strong genetic influence which was estimated to be between 39 and 65%, in certain forms of OA [Spector et al., 1996].

The heritability of primary generalised OA without underlying skeletal involvement, termed familial primary generalised OA (FOA) [Meulenbelt et al., 1997], has been extensively debated. Evidence of defective collagen genes in some persons with OA [Ala-Kokko et al., 1990; Knowlton et al., 1990; Prockop et al., 1997] was contrary to the general belief by rheumatologists that generalised OA was not a heritable condition. Evidence for a genetic predisposition in OA was provided by the observations of Kellgren et al. [1963] and Doherty et al. [1983]. These investigators found that OA associated with enlargement of the terminal joints of the fingers (Heberden's nodes)

was sex-influenced, being dominant with full penetrance in females, but recessive in males. Kellgren and Moore [1952] found clinical evidence of a direct association between Heberden's nodes and OA of other joints including the spine and the knees. Their findings were confirmed by a radiological study on a random sample of a Lancashire population [Kellgren and Lawrence, 1958].

Support for COL2A1 involvement, and thus a genetic basis to FOA, was provided by Vikkula et al. [1989] and Palotie et al. [1989] who demonstrated that DNA markers from the COL2A1 locus cosegregated with the condition in two kindred. However, despite extensive mutation screening of COL2A1, they were unable to identify a pathogenic mutation. Many molecular investigations have been concerned with determining the involvement of the COL2A1 gene in which mutations have been identified in some families with early onset OA associated with a mild chondrodysplasia [Pun et al., 1994; Bleasel et al., 1995]. Other investigations seeking a genetic predisposition for Heberden's nodes in a patient cohort in the UK [Priestley et al., 1990] and in a Finnish isolate [Vikkula et al., 1993a], however, revealed no definite association between this condition and a number of DNA polymorphisms in the COL2A1 gene.

In a recent investigation [Meulenbelt et al., 1997], linkage analysis for FOA in a large Dutch family of Jewish descent was extended to other candidate genes which are important for the integrity of articular cartilage. The findings resulted in the exclusion

of the collagen genes COL2A1, COL9A1, COL9A2, COL11A1, COL11A2, and the non-collagenous genes COMP, CRTL1, CRTM, CCAL2 and matrix metalloproteinase 3 (MMP3), in that kindred. Other genes which were tested but not excluded were COL9A3, DCN, LOX (the lysyl oxidase gene which is defective in Cutis Laxa and PLOD (the gene for procollagen-lysine, 2-oxoglutarate 5-dioxygenase which is defective in type VI Ehlers-Danlos syndrome).

The present study involves a candidate gene-based analysis of a large South African family of Jewish descent with FOA. The affected members of this kindred, manifesting with knee pain and Heberden's nodes, were investigated by clinicians in the Department of Human Genetics at UCT. Although a limited number of family members were available for study, they were deemed sufficient to provide an indication of a possible aetiology for FOA in the kindred.

6.2. METHODS AND MATERIALS

(i) The Family

A pedigree of the 59 membered, 4-generation kindred is shown in Fig. 6-1. The family comprises 18 clinically confirmed affected individuals, and three who were anecdotally thought to be affected. The youngest affected individual (IV-6) was 25 years of age at the time of clinical evaluation. Individuals were classified as 'affected' on the basis of a

long-standing history of knee and ankle pain, Heberden's nodes, and radiological evidence. Detailed clinical data and radiolographs were obtained from three of the affected members (III-18, III-26 and IV-6). Informed consent was provided by all participants prior to inclusion in the study. Blood specimens were collected from these persons together with three other affected relatives (II-18, III-7 and III-10) and five completely asymptomatic adult relatives (III-2, III-17, III-28, IV-4, IV-5 and IV-7) who were accorded an 'unaffected' status. The designated affection status of the deceased individuals in the family was surmised information from their close living relatives and from the transmission of the phenotype to their living offspring.

The affected individuals in this kindred presented with pain in the knees and ankles in their early twenties. Involvement of the terminal joints of the fingers subsequently developed and Heberden's nodes were present in the two older individuals (III-18 and III-26) at the time of clinical assessment. There was no clinical or radiological evidence of an associated chondrodysplasia in the affected persons who were investigated [Beighton, personal communication]. Furthermore, there was no evidence of stunted stature or brachydactyly which would categorise the condition as MED, and no evidence of eye or hearing abnormalities which would group them with the Stickler syndrome. Only findings which were typical of a generalised OA were observed. The OA in the family was progressive and lead to severe physical handicap in the older individuals. Radiographs of the knees, ankles and spine showed narrowing of joint spaces and large bony spurs (osteophytes), which were indicative of OA (Fig. 6-2,a-d). The affected persons had moderate OA of the hip. Radiographs

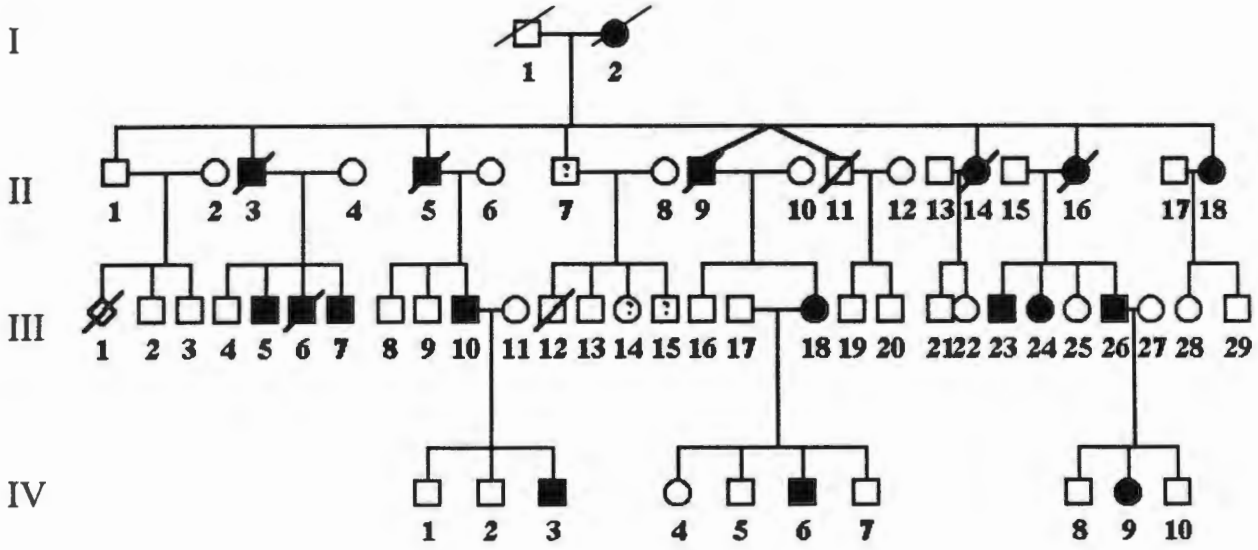

Fig. 6-1. Pedigree of a South African kindred manifesting with primary generalised OA and Heberden's nodes, inherited as an AD trait.

of the hands showed a narrowing of the joint spaces and osteophytes in the distal finger joints (Fig. 6-2e).

(ii) Molecular Investigations

Individual III-18 presented herself and her affected son, aged 25 years (IV-6) at the Department of Human Genetics, UCT, for a genetic evaluation. Prior to molecular investigation, other accessible family members who were recruited for this study included individuals II-18, III-2, III-7, III-10 and III-28. In order to undertake an extended linkage analysis peripheral blood samples were collected, with informed consent, by venipuncture from all available affected and unaffected family members. A limited number of relatives were available for study since most of them had emigrated and currently reside in distant countries. A total of twelve family members were accessible and finally used for linkage analysis.

The primary candidate for this study was COL2A1 to which FOA has previously been linked [Vikkula et al, 1989]. Eighteen additional candidate genes were screened using closely linked microsatellite markers, the majority of which were selected from the published genetic maps [Gyapay et al., 1994, Dib et al., 1996]. The markers (Ch. 2, Table 2-II) had an average separation of 1-10cM as determined by use of the Genome database (http://www.gdb.org) and the Généthon linkage map [Dib et al., 1996]. The candidate genes which were screened were COL6A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, AGC1, DCN, FMOD, LUM, MMP3, COMP, CCAL2 and CRTL1. In addition, the non-articular collagen marker, CRTM, was also screened.

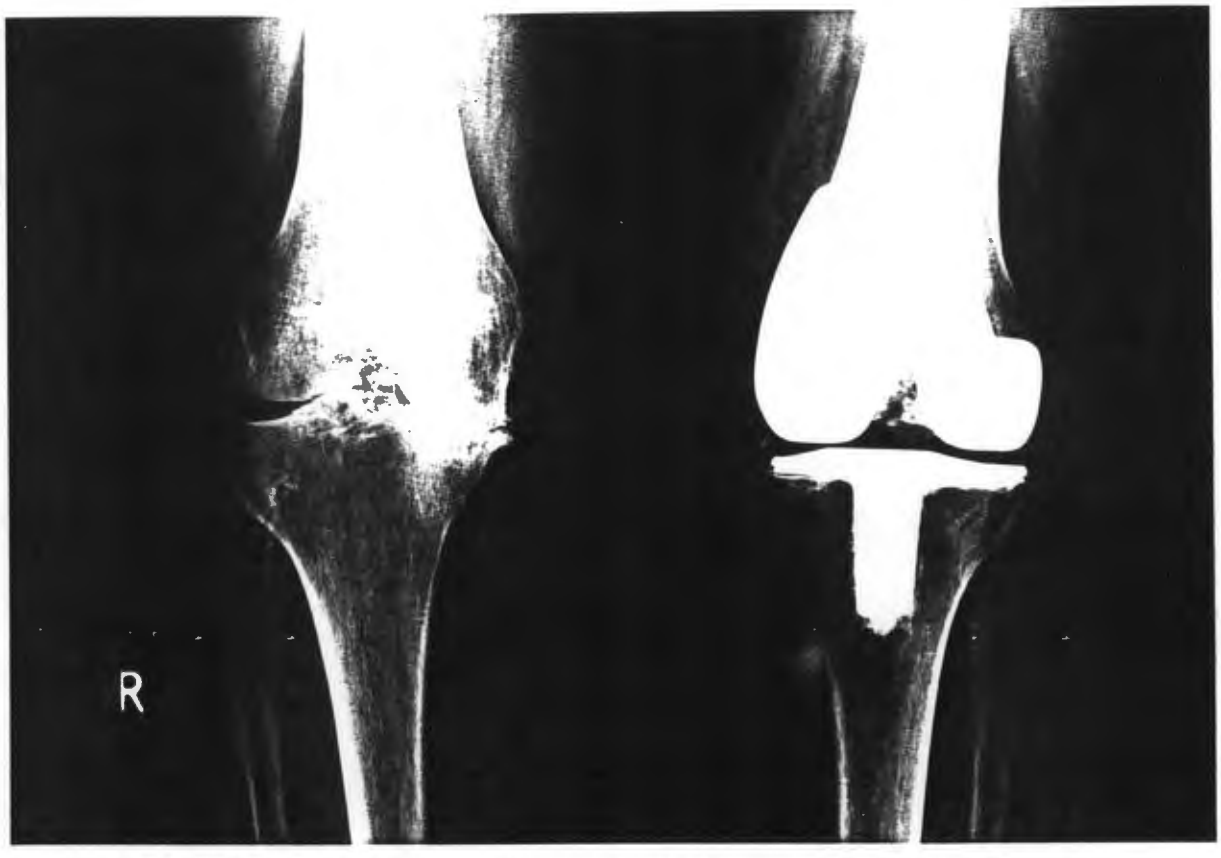

Fig. 6-2(a). FOA: Antero-posterior radiographs of the knee joints of an affected female, aged 56 years (III-18). Marked narrowing of the joint space and periarticular sclerosis are evident in the right knee. Prosthetic joint replacement has been undertaken in the left knee.

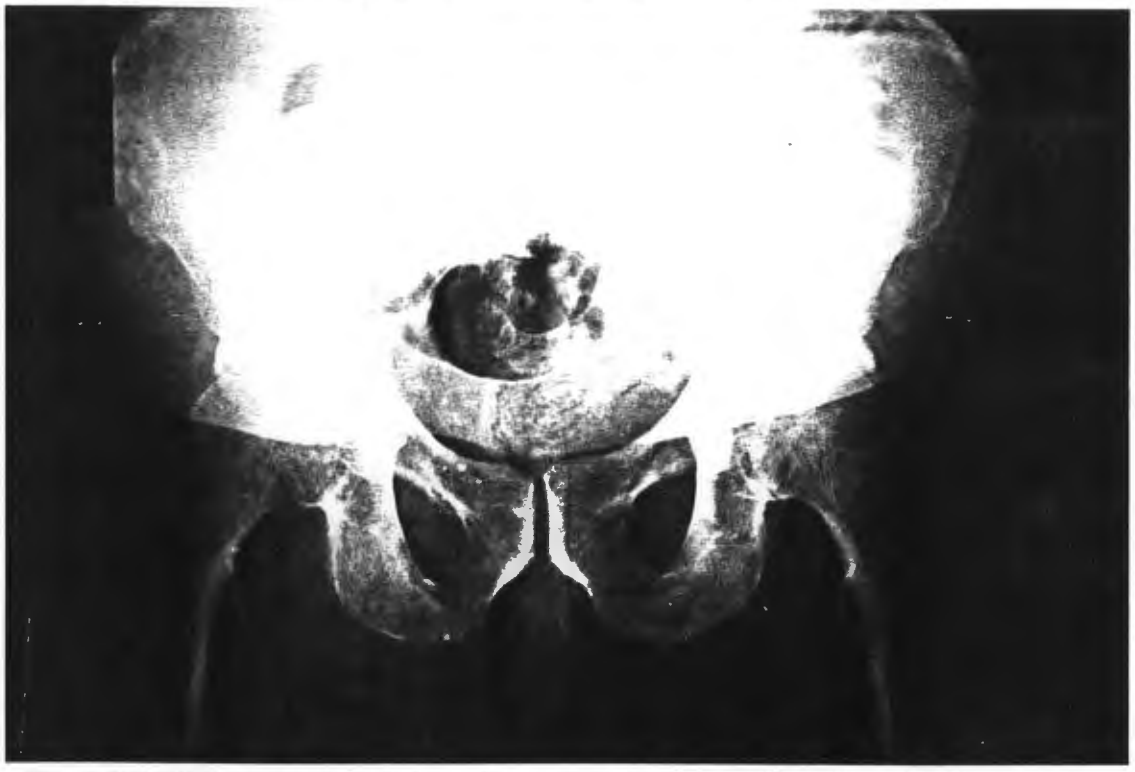

Fig. 6-2(b). FOA: Antero-posterior radiograph of the pelvis of an affected male aged 66 years (III-26). The hip joints are irregular and sclerotic.

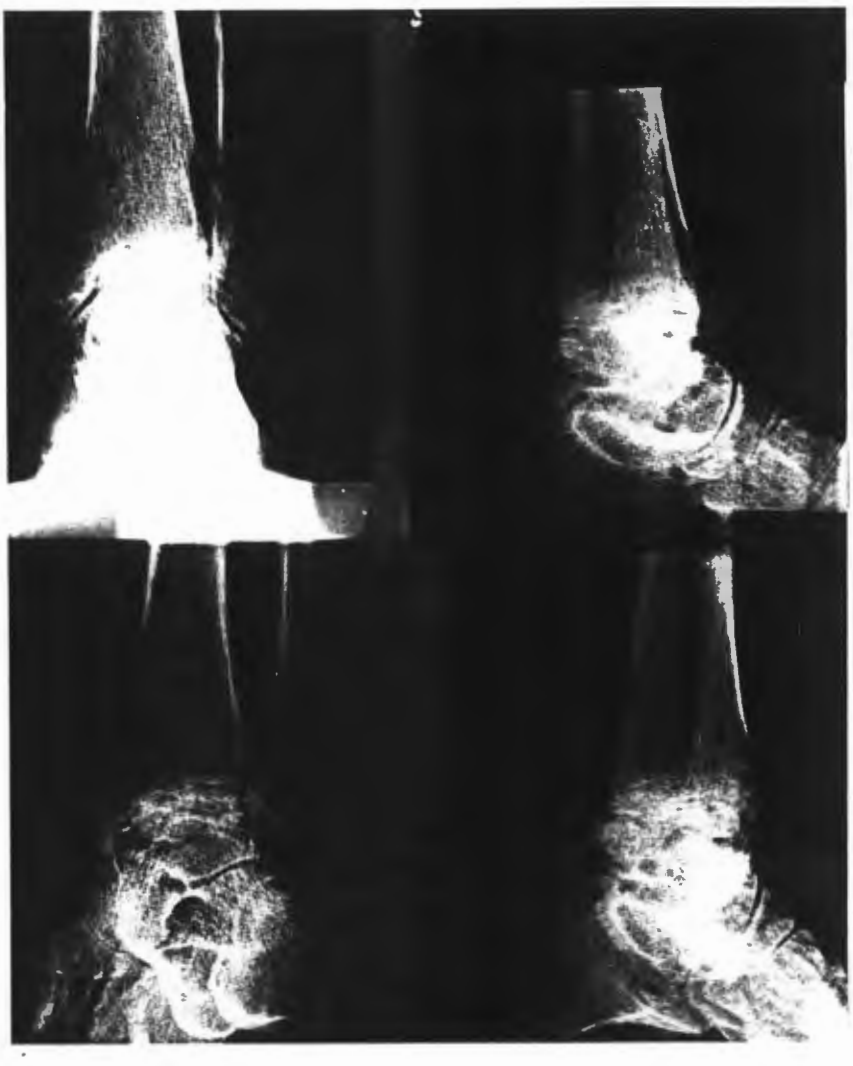

Fig. 6-2(c). FOA: Radiographs of the ankle of an affected female aged 56 years (III-18).The joint space is narrow and irregular with large osteophytes and considerable sclerosis.

Fig. 6-2(d). FOA: A lateral radiograph of the spine of an affected male aged 66 years (III-26).The intervertebral spaces are narrowed and uneven; endplate sclerosis and anterior osteophytes are evident.

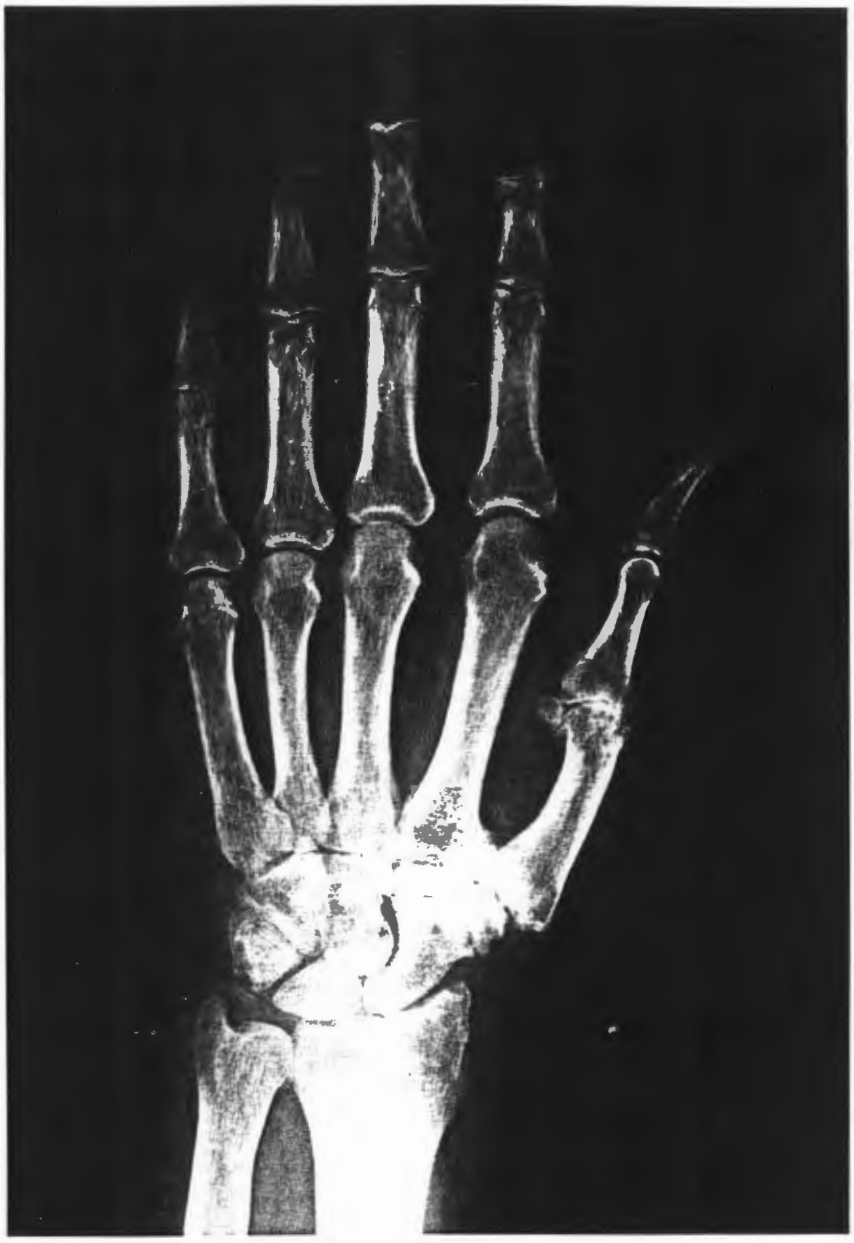

Fig. 6-2(e). FOA: A radiograph of the hand of an affected male aged 66 years (III-26). The middle and distal interphalangeal joints are narrow and irregular with significant osteophytes. The carpo-metacarpal and metacarpal-phalangeal joints in the thumb are similarly affected.

Following the preliminary study, additional molecular investigations required a further eight polymorphic microsatellite markers which span the COL11A1 region and which were selected off the Généthon human linkage map [Gyapay et al., 1994, Dib et al., 1996]. These markers included D1S497, D1S420, D1S486, D1S429, D1S485, D1S495, and D1S457 (Table 6-II). A final investigation which was undertaken in collaboration with Dr Leena Ala-Kokko (Finland), involved screening the family with two polymorphic intragenic markers for COL11A1 described by her group (personal communication) viz. a tetranucleotide repeat in intron 16: F-5' ggccctcgaggcgtccag-3'/ R-5' cagcatgattaagcgaagtgac-3' and a trinucleotide repeat in intron 17: F-5' cctgtggcagttcactctgg-3'/ R-5' acctttgtcacctggcagac-3'.

Genomic DNA was extracted from whole blood using standard techniques (Appendix, section 1.1.) and the DNA was genotyped using PCR of microsatellite markers (Appendix 1, section 2.1.2.). Alleles were separated according to size on PAGE gels, dried at 80°C, and exposed at -70°C for autoradiography (Appendix 1, section 2.1.3.).

Linkage analyses were performed using the LINKAGE package Version 5.03 [Lathrop and Lalouel, 1984] and the map order from the Généthon map of microsatellite markers for 1p21. In all analyses the number of alleles for each marker was reduced to five, without loss of information in the family, in order to accommodate the computer program's limitations for numbers of alleles. Male and female recombination rates were taken to be equal. The condition was assumed to be autosomal dominant and in the absence of data on penetrance and expressivity, a value of 0.9 for penetrance was used

in order to allow for a minor fraction of non-penetrance that may be encountered in this disorder. A gene frequency of 0.1 was assumed for the disease locus.

6.3. RESULTS

Twelve members of this family were genotyped with thirty polymorphic markers (nine of them being intragenic) from eighteen candidate gene loci. Two-point LOD scores and recombination fractions are shown in Table 6-I. On the basis of $Z < -2.00$ at $\theta=0.05$ [Lester et al., 1990], COL2A1, COL6A3, COL9A1, AGC1 and MMP3 could be excluded from causing the FOA phenotype in the South African kindred. Meiotic recombinations with intragenic markers for COL6A2, COL10A1, COL11A2, CRTL1, DCN and CRTM suggest that these genes could also be excluded as FOA candidates (although intragenic recombinations have been known to occur within a mutant gene). The remaining genes which were investigated viz. COL9A2, COL9A3, COMP, LUM, FMOD and CCAL2 genes could not be excluded by two-point linkage analysis.

No recombinations were observed between the FOA phenotype and markers, D1S223 and D1S206, located about 5cM away from the COL11A1 gene on 1p21. Two point LOD scores revealed a positive LOD score of 1.31 at theta=0.00 (D1S223) and 1.94 at theta=0.00 (D1S206), suggesting that COL11A1 could not be excluded as the locus for FOA in this family. Further investigation of COL11A1 was undertaken using additional markers flanking the locus. Disease-associated alleles were determined for III-18 and her son IV-6 and the haplotype which appeared to be segregating with the condition was constructed (Fig. 6-3). The parental haplotypes of five individuals, II-18, III-2, III-

7, III-10 and III-26, could not be determined because their parents were deceased. For this reason, the phase of the OA phenotype in these additional individuals was determined by looking for the same haplotype which appeared to be segregating with the condition in III-18 and IV-6. Similar haplotypes for the region between D1S420 and D1S495 were identified in the affected individuals II-18, III-7 and III-10 but not in the unaffected person, III-2 (Fig. 6-3).

Meiotic recombinations were observed between the OA phenotype and the markers within and flanking the COL11A1 gene, thereby excluding it as a candidate for FOA in this kindred (Fig. 6-3, Table 6-II). Moreover, two recombination events in the affected individual, III-26, allowed the delineation of a candidate region to a 3-cM interval spanning D1S223 and D1S206 which is approximately 5cM away from the COL11A1 locus.

Table 6-I. Two point LOD scores between FOA in the South African kindred and polymorphic microsatellite markers from the candidate gene loci.

GENE	[CA]n marker	0.00	0.05	0.1	0.2	0.3	0.4	0.45
COL2A1	D12S361	-∞	-2.66	-1.58	-0.64	-0.23	-0.05	-0.01
	VNTR	-∞	-2.24	-1.42	-0.69	-0.34	-0.14	-0.06
COL6A2	[CA]n	-∞	-0.50	-0.05	0.21	0.18	0.06	0.01
	D21S1446	-∞	-0.95	-0.49	-0.16	-0.05	-0.01	-0.00
COL6A3	D2S338	-∞	-1.17	-0.67	-0.27	-0.10	-0.02	0.00
	D2S345	-∞	-2.25	-1.38	-0.59	-0.23	-0.05	-0.01
COL9A1	509- 8B2	-∞	-2.31	-1.28	-0.44	-0.12	-0.01	-0.00
	509-12B1	-∞	-2.36	-1.30	-0.48	-0.15	-0.03	-0.01
COL9A2	MYCL-1	-∞	0.94	0.99	0.78	0.47	0.18	0.07
	D1S1598	-∞	0.85	0.94	0.81	0.55	0.27	0.13
COL9A3	D20S64	-∞	-1.76	-1.00	-0.37	0.11	-0.01	0.00
COL10A1	RFLP	-∞	-0.79	-0.34	-0.06	0.00	-0.01	-0.01
COL11A1	D1S223	1.31	1.16	1.02	0.74	0.47	0.22	
	D1S206	1.94	1.73	1.51	1.06	0.62	0.23	
COL11A2	D6S291	-∞	-0.14	0.27	0.23	0.11	0.02	0.00
	D6S273	-∞	-1.81	-1.05	-0.45	-0.20	-0.08	-0.03
	RFLP-*Msp*I	-∞	-1.10	-0.62	-0.25	-0.12	-0.05	-0.02
AGC1	VNTR	-∞	-2.20	-1.16	-0.31	0.01	0.08	0.06
CRTL1	VNTR	-∞	-0.84	-0.37	-0.06	-0.01	0.01	0.00
COMP	D19S215	-∞	-1.24	-0.74	-0.32	-0.12	-0.02	0.00
	D19S212	-∞	-0.46	-0.23	-0.06	-0.01	-0.00	0.00
LUM	D12S101	-∞	-0.35	0.05	0.22	0.14	0.01	-0.02
	D12S1064	-∞	0.05	0.14	0.04	-0.11	-0.15	-0.01
DCN	$[CA]_n$	-∞	-1.22	-0.75	-0.43	-0.33	-0.22	-0.12
FMOD	D1S1660	-∞	-1.86	-1.09	-0.44	-0.16	-0.04	0.00
	D1S306	-∞	-0.92	-0.46	-0.14	-0.04	-0.01	0.00
CRTM	VNTR	-∞	-0.39	-0.19	-0.05	-0.01	0.00	0.00
MMP3	D11S898	-∞	-2.59	-1.53	-0.63	-0.23	-0.05	-0.01
CCAL2	D8S539	-∞	-0.03	0.13	0.14	0.07	0.02	0.00
	D8S521	-∞	-0.41	-0.02	0.16	0.13	0.06	0.03

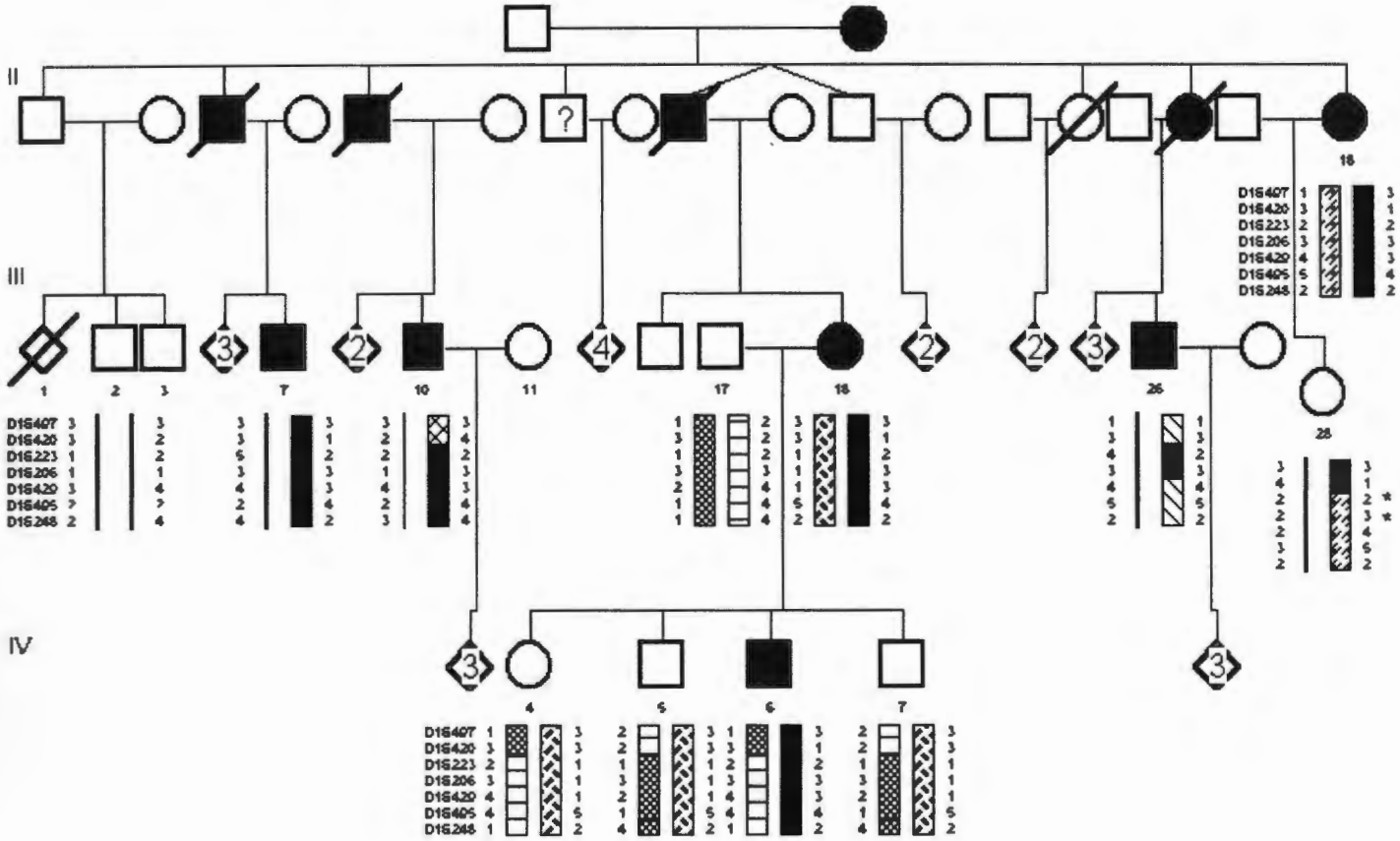

Fig. 6-3. An abridged pedigree of the South African kindred. The genotypes for markers from the COL11A1 locus are indicated below each individual. Haplotypes are depicted as shaded bars, the disease haplotype being shaded in black. The haplotypes for III-2 are indeterminate, as are the genotypes (marked with *) for individual III-28.

Table 6-II. Microsatellite markers selected from the Généthon map which were used to screen the South African kindred with FOA at the COL11A1 locus. The genetic distances between the markers, in the order of appearance on the map, are indicated. According to the Stanford Human Genetic Center [http://www.shgc.stanford.edu/mapping/rh/mapsv2/search1.html], the COL11A1 gene (indicated by intronic markers 16 and 17) lies between the markers D1S495 and D1S248.

GENETHON [CA]n MARKER	GENETIC DISTANCE (cM)	0.00	0.05	0.10	0.20	0.30	0.40
D1S497	0	-∞	-1.38	-0.80	-0.30	-0.09	0.02
D1S420	3	-∞	-0.59	-0.14	0.14	0.13	0.04
D1S223	0	1.31	1.16	1.02	0.74	0.47	0.22
D1S206	4	1.94	1.73	1.51	1.06	0.62	0.23
D1S429	0	-∞	0.57	0.71	0.68	0.51	0.27
D1S495	1	-∞	0.50	0.60	0.51	0.31	0.11
Marker 16		-∞	-0.71	-0.28	-0.02	-0.01	-0.03
Marker 17		-∞	-2.79	-1.71	-0.78	-0.35	-0.12
D1S248	1	-∞	-0.44	-0.22	-0.08	-0.03	-0.01

6.4. DISCUSSION

In a candidate gene screen for linkage of inherited primary generalised OA (FOA) without an underlying skeletal dysplasia, the genes encoding eighteen cartilage components were screened. Twelve family members were studied, six of whom were affected with the condition.

A significant LOD score of <-2.00 at θ=0.05 in this study has demonstrated that the COL2A1 gene, which has previously been associated with FOA [Vikkula et al., 1989], is not involved in the aetiology of FOA in this South African kindred. On the same basis, the present findings indicate that the COL6A2, COL9A1, COL10A1, COL11A1, COL11A2, AGC1, CRTL1, DCN and the CRTM genes are also not primarily involved in this condition. The other candidate genes which were investigated could not, however, be excluded from involvement; total exclusion would require that either intragenic markers be used or that each gene be screened for a pathogenic mutation if the family is not sufficiently informative for linkage.

The present findings do, however, suggest that a candidate gene for FOA may be localised to 1p21, to a genomic region between D1S223 and D1S206. These markers are thought to lie at least 5cM telomeric to the COL11A1 gene and have previously been used to demonstrate linkage between COL11A1 and a chondrodysplasia phenotype [Richards et al., 1996]. The COL11A1 gene has recently been shown to harbour a mutation which results in the chondrodysplasia, Stickler syndrome type 2

[Snead et al., 1994; Richards et al., 1996]. In the South African kindred, however, meiotic recombinations using highly informative intragenic markers for COL11A1 indicated that this gene did not harbour the pathogenic mutation. It is possible, therefore, that a gene for FOA, as yet unidentified, may be localised to the 1p21 region, approximately 5cM telomeric to the COL11A1 gene. The South African family under investigation, however, comprised only a small number of people who were accessible for study, and it is therefore possible that the apparent lack of recombination in the 1p21 region may be spurious. As mentioned in Ch. 4 (section 4.4) this phenomenon is not uncommon when performing linkage analysis in small families and can falsely imply an association between a marker and the disorder in such an investigation. It is therefore possible that molecular investigations of further candidate genes may also yield non-recombinations in this small kindred; mutation analysis of each gene, where possible, will therefore be necessary to verify the linkage results.

The findings in this South African kindred support those of Meulenbelt et al.[1997] who demonstrated genetic heterogeneity for FOA. In order to achieve definitive results, it would, however, be necessary to increase the number of family members for this investigation. The limitations in dealing with a condition such as this, which is relatively mild until mid-adulthood, is that the parents of the affected individuals may be deceased by the time of clinical evaluation, thus placing a constraint on linkage analysis. It may therefore be necessary to inform these kindred of the need to undertake molecular investigations presymptomatically, thereby providing risk estimates for family members, and thus enable affected individuals to plan ahead. The

recognition of a specific molecular basis for the disorder will facilitate presymptomatic diagnosis. In this way, although the condition is not life-threatening, the knowledge of having a predisposition to pain and discomfort which would impose limitations on everyday activities and which may necessitate surgical procedures at some stage, could enable individuals to plan ahead for suitable employment and lifestyle.

Apart from this potential benefit, the FOA phenotype probably is a closer approximation to the generalised 'sporadic' OA, which is a major health and socioeconomic burden in any country, than the secondary forms of OA. Identification of the determinant gene(s) for FOA may realistically contribute towards the understanding of the genetic and biological mechanisms which play a role in age-related generalised OA. The work presented here may be considered preliminary but should provide impetus for the further recruitment of as many members of the kindred as possible, and probably to participate in an international collaborative mapping exercise for FOAs.

CHAPTER 7:
MSELENI JOINT DISEASE (MJD)

7.1. INTRODUCTORY BACKGROUND

Mseleni joint disease (MJD) is an unusual form of precocious and generalised OA which was identified in several hundred persons within a small area in the remote, rural Mseleni region in Northern Kwa-Zulu Natal, South Africa. The condition has its onset in mid-childhood and is characterised by progressive degenerative changes in the hip joint and, to a lesser extent, the spine and other weight-bearing joints [Lockitch and Fellingham, 1973; Solomon et al., 1976]. These changes lead to secondary OA and serious disability usually from the third decade of life [du Toit, 1979]. In addition to the pain and handicap experienced by affected persons, MJD places a heavy burden on the state, including the costs of hospitalization for prosthetic joint replacement and disability pensions.

Since individuals with MJD may have vertebral anomalies [Lockitch, 1974], the condition may be regarded as a form of spondylo-epiphyseal dysplasia (SED). Another peculiar feature of MJD is the gross dwarfism which occurs in about 0.1% of the Mseleni population as a whole [Viljoen et al., 1993]. Apart from brachydactyly

and shortened long bones, these individuals share the clinical and radiographic phenotype of conventional MJD.

MJD initially came to general medical attention in the early 1970's. The findings of a genealogical and radiological study of 5 large kindred, was suggestive (but not conclusive) of an autosomal dominant pattern of inheritance [Lockitch, 1974]. The observation that the condition was localised within a small area, and the lack of an apparent Mendelian inheritance pattern within affected kindreds, suggested that environmental factors might be responsible for the phenotype. Early investigations on the affected MJD population, however, failed to reveal significant abnormalities in biochemical factors between affected and control populations [Burger et al., 1973]. No disturbances of blood group frequencies were detected. Biochemical studies of the serum and food and water supplies failed to reveal any differences between affected and control groups [Lubbe et al., 1973]. In contrast, evidence of the hip dysplasia among affected relatives supported the concept of a genetic basis for this disorder.

Interestingly, the MJD phenotype resembles that of a severe, progressive form of SED found in two small isolates of jungle-dwellers in Karnataka, southern India [Agarwal et al., 1994]. This latter condition, termed Handigodou joint disease (HJD), has a similar clinical and radiological phenotype as MJD. The presence of unaffected communities from culturally different caste groups living in the same area, is evidence against an acquired aetiology. Pedigree data in HJD are suggestive of autosomal dominant inheritance [Agarwal et al., 1997].

Because of a lack of Mendelian inheritance, MJD should be considered a complex trait [Lander and Schork, 1994]. Complex traits are often polygenic and, due to features such as incomplete penetrance, phenocopy and genetic heterogeneity, these traits may not be amenable to simple segregation analysis. Lander and Schork [1994] described four methods of genetically dissecting complex traits which comprise linkage analysis, allele-sharing methods, association studies and experimental crosses in animal models.

Linkage analysis is based on family studies and attempts to explain the inheritance of phenotypes and genotypes in a pedigree. Allele-sharing methods involve the study of affected relatives in a pedigree to determine how often a chromosomal region is inherited from a common ancestor. The simplest form of allele sharing is sib pair analysis which has played a major role in the localisation of the gene for type I diabetes and the angiotensinogen gene for hypertension [Jeunemaitre et al., 1992], amongst others. Association studies are "case-controlled", based on comparing allele frequencies in unrelated affected individuals with unaffected individuals from the same population. These studies do not concern inheritance patterns. A positive association between a gene and marker can be established if an allele occurs at a significantly higher frequency among the group of affected people but not in the control group. Furthermore, in a genetically isolated population, linkage disequilibrium could prove to be a powerful mapping tool and could also identify a common ancestor for disease-causing alleles. Of the four methods mentioned above, association studies are the most suited to the present genetic dissection of Mseleni

joint disease and in particular to testing the hypothesis that MJD is due to a founder-effect. Earlier investigations using segregation analysis [Lockitch, 1974] did not shed any light on a suspected founder-effect theory for MJD.

It is of crucial importance to association studies that a suitable "control" group of individuals is selected. Family-based association studies and the development of "internal controls" or AFBACS (affected family-based controls) provides a powerful strategy for identifying suitable controls [Thomson, 1995]. The AFBACS are the parental alleles which are never transmitted to an affected child or to an affected sib pair. This method of ascertainment provides an unbiased estimate of the control alleles for markers which are closely linked to a gene. False associations which result from ethnic admixture, migration and population stratification can therefore be avoided. The use of AFBACS and sib pair analysis led to the association of IDDM and an allele of the insulin gene [Thomson, 1989] where haplotype-sharing data had showed no evidence of linkage [Spielman et al., 1989]. In a random-mating population with no stratification, no admixture and no migration, for θ=0.00, the family-based study is equivalent to a case-controlled study.

The Department of Human Genetics, UCT, first became involved with the Mseleni population over two decades ago. The epidemiological findings at that time were inconclusive [Lockitch, 1974]. More recently, over a period of three years (1993-1995), staff members visited the Mseleni district in order to reassess the genetics and to obtain biological material for a pilot molecular genetic investigation of MJD. Blood specimens were obtained mostly from unrelated individuals because complete

families were not always accessible. In many instances the parents of affected individuals were deceased, limiting the availability of nuclear families and sibpairs.

The present investigation was designed with the objective of finding an indicator of a possible aetiology for the MJD phenotype. It was envisaged that the findings would facilitate the implementation of a large scale study of the Mseleni population and the individuals affected with MJD. In order to determine whether MJD had a genetic component, in the present study, four different avenues of research were undertaken. Firstly, an association between MJD and the Human Leukocyte Antigen (HLA) complex was examined since other complex disorders such as rheumatoid arthritis [Mody et al., 1989] and diabetes [Copeman et al., 1995] have associations with a specific haplotype of HLA. Secondly, articular cartilage specimens were examined histologically for structural abnormalities which could be indicative of the underlying biochemical and genetic defect. Thirdly, allele frequencies in the affected population were compared with those of unaffected individuals from the same geographical area. Some of these findings which are incorporated into this chapter have been published during the course of this project [Ballo et al., 1996]. Lastly, as COL2A1 is known to be defective in numerous chondrodysplasias which have OA as a component, mutation analysis of the gene was undertaken in an affected person with the typical SED phenotype and in a dwarfed individual from the Mseleni community, in order to identify possible disease-predisposing changes.

7.2. METHODS AND MATERIALS

(i) The Mseleni district

This area has been described elsewhere [Lockitch, 1974] and is briefly summarised here. The Mseleni region, falls in the coastal belt of subtropical forest or dense evergreen bush. The Mseleni area is about 13x18km and is situated in the Ubombo district of northern Kwa-Zulu Natal. It lies midway between St. Lucia (south) and Kosi Bay (north), on the western shore of Lake Sibaya, which is about 50km south of the Mozambique/ South Africa border.

(ii) The Mseleni Population

Affected group

The samples for the pilot study initially comprised 30 unrelated, unaffected MJD persons (13 males and 17 females) with an age range of 40-90 years. At a later stage, a further 17 affected individuals became available for study. Affected individuals were identified by virtue of having widespread involvement of the epiphyses and vertebral bodies, and, where possible, the diagnosis was confirmed radiologically.

In many instances the pattern of inheritance could not be determined because of complicated pedigree structures. In some instances, families had either both parents affected, all of the offspring affected or marriages had occurred between affected individuals of normal stature and individuals whose growth was severely stunted (i.e. dwarfed individuals).

Control group

The Mseleni population is geographically isolated. The socioeconomic status of the population, and lack of migrational incentives has resulted in the population being relatively homogeneous. This homogeneity is advantageous for identifying a control group of individuals that is well-matched to affected individuals for association studies. Furthermore, it is likely that random-mating is occurring in this population and genetic markers appear to be in Hardy-Weinberg equilibrium [Lockitch, 1974]. For the aforementioned reasons, and in accordance with family-based AFBAC association studies [Thomson, 1995], the unaffected individuals from the greater Mseleni region were considered to be a suitable control group for this study. The selected group for the pilot study comprised 40 unrelated individuals of age >30 years. Only healthy subjects who had no family history of MJD were included in the control group.

(iii) HLA Association

HLA is the major histocompatibility system which plays a dominant role in the genetic control of a variety of immune functions that influence disease susceptibility and resistance [Shreffler, 1977]. The region is particularly polymorphic comprising 4 different loci, each having between 6 and 22 different alleles [Payne, 1977].

In order to determine whether an association exists between the HLA complex and MJD, blood specimens from affected and unaffected individuals were tested with probes for the class II HLA antigens, DRβ and DQβ [Ballo et al., 1996]. The study was undertaken at the Provincial laboratory for Tissue Immunology at the UCT

Medical School. PCR products were dot-blotted onto nylon membranes and probed for specific alleles. Hybridisation patterns were visualised by chemiluminescence according to the supplier's protocol [*Boehringer Mannheim*, Germany].

(iv) Histological Analysis

Histological examination of cartilage tissue by means of electron microscopy has proven very useful in providing information about the ultrastructure of cartilage in persons affected with various forms of chondrodysplasia with an associated OA [Diab et al., 1994; Winterpacht et al., 1993].

In order to determine whether any specific ultrastructurally identifiable component of cartilage from individuals with MJD had been altered, cartilage biopsies were sectioned for electron microscopy. The present study was performed in conjunction with Dr Doug Keene at the Research Laboratory, Shriner Hospital for Crippled Children, Portland, Oregon, USA. Femoral heads excised from affected adults requiring prosthetic hip joint replacement operations, became available for laboratory investigation. Six specimens of this type were collected following operation and prepared for electron microscopy.

(v) Molecular Analysis: Comparison of allele frequencies at candidate gene loci

In a non-Mendelising disorder such as MJD, the trait may be multifactorial, being caused by the interaction of numerous genes with each other or with the environment. Classical linkage analysis is not suitable for detecting susceptibility

loci in these disorders, since it assumes that there is only one disease locus and relies on the use of an estimated value for penetrance of the trait. A more appropriate approach for the localisation of the gene(s) for a complex trait would be affected sibpair (ASP) analysis which tests for the sharing of alleles between sib pairs and has proven to be very powerful in mapping disease susceptibility loci [Weeks and Lathrop., 1995; Dyment et al., 1997; Nair et al., 1997]. The ASP method does not require the mode of inheritance to be known, and does not require extended families for investigation. This was important in MJD as many family members were inaccessible and therefore limited numbers of people were available for study. Despite several trips to the Mseleni district, however, it was not possible to obtain the appropriate numbers of sibpairs which are required to validate the results of the ASP analysis. For this reason, a different level of association was investigated, based on the hypothesis that MJD may be due to mutations in one or more candidate genes. The frequencies of alleles at the different candidate gene loci were determined to see whether any alleles were more frequent in affected individuals than in normal controls from the same geographic region.

Venous blood (10ml) was obtained by venipuncture. Genomic DNA was extracted using the Genomix^{TM} blood scale-up kit [*Talent*, Italy] (Appendix 1, section 1.1.). Synthetic oligonucleotide pairs for the intragenic and closely linked microsatellite markers (Table 7-I), were obtained commercially [*Research Genetics*, USA] or synthesized locally at the UCT. PCR reactions, to which d-CTP^{32} had been added, were performed in a total reaction volume of 10ul (Appendix 1, section 2.1.2.). Reaction products were resolved on 6% PAGE gels

gels which were dried at 80°C and autoradiographed O/N at -70°C (Appendix 1, section 2.1.3.).

Allele frequencies and heterozygote frequencies were calculated by allele counting. Concordance of genotype frequencies with Hardy-Weinberg equilibrium was tested by χ^2 goodness-of-fit test [Emery, 1986].

(vi) Mutation screening of COL2A1.

Mutations in the COL2A1 gene have been identified in several chondrodysplasias in which OA is a component or consistent complication (see Ch. 4, Table 4-I). The present study could therefore not exclude the possibility of COL2A1 being responsible for the development of, or of it harbouring a polymorphism which predisposes the carrier to, the MJD phenotype.

In collaboration with Dr Jarmo Korkko of the Allegheny University of the Health Sciences, Philadelphia, USA, the COL2A1 exons in two affected individuals, one who had classical MJD and one who was dwarfed, were screened using the intronic primers tabulated in Ch. 3 (Table 3-II) and the CSGE procedure (see Appendix 1, section 2.2.3).

7.3 RESULTS

HLA association

No significant differences in the allele frequencies ($p>0.05$) of class II antigens, DRβ and DQβ, were found between affected individuals and healthy control subjects from the Mseleni district [Ballo et al., 1996].

Histological Analysis

Electron microscopic investigations of the cartilagenous region of the femoral heads revealed a large excess of broad-banded structures around the articular chondrocytes (Fig.7-1). These aggregates had a cross-striated appearance which is characteristic of collagen fibrils rich in type VI collagen [Roncuzzi et al., 1990].

Molecular Analysis: Comparison of Allele frequencies

Allele frequencies in the unrelated MJD individuals and the normal controls are tabulated and compared with reported figures [Research Genetics] in Table 7-I. The allele frequencies for the candidate gene markers were mostly similar to that of the control group ($p<0.05$; $\chi^2 > 3.48$ with 1 degree of freedom [Emery, 1986]). The heterozygosity of the markers was generally similar between the two groups, and the published frequencies. In those instances where the values differed markedly from the literature (COL9A3, COL10A1-D6S474, COL10A1-RFLP/HindIII, D8S539, and D19S215) the control and affected groups had similar frequencies.

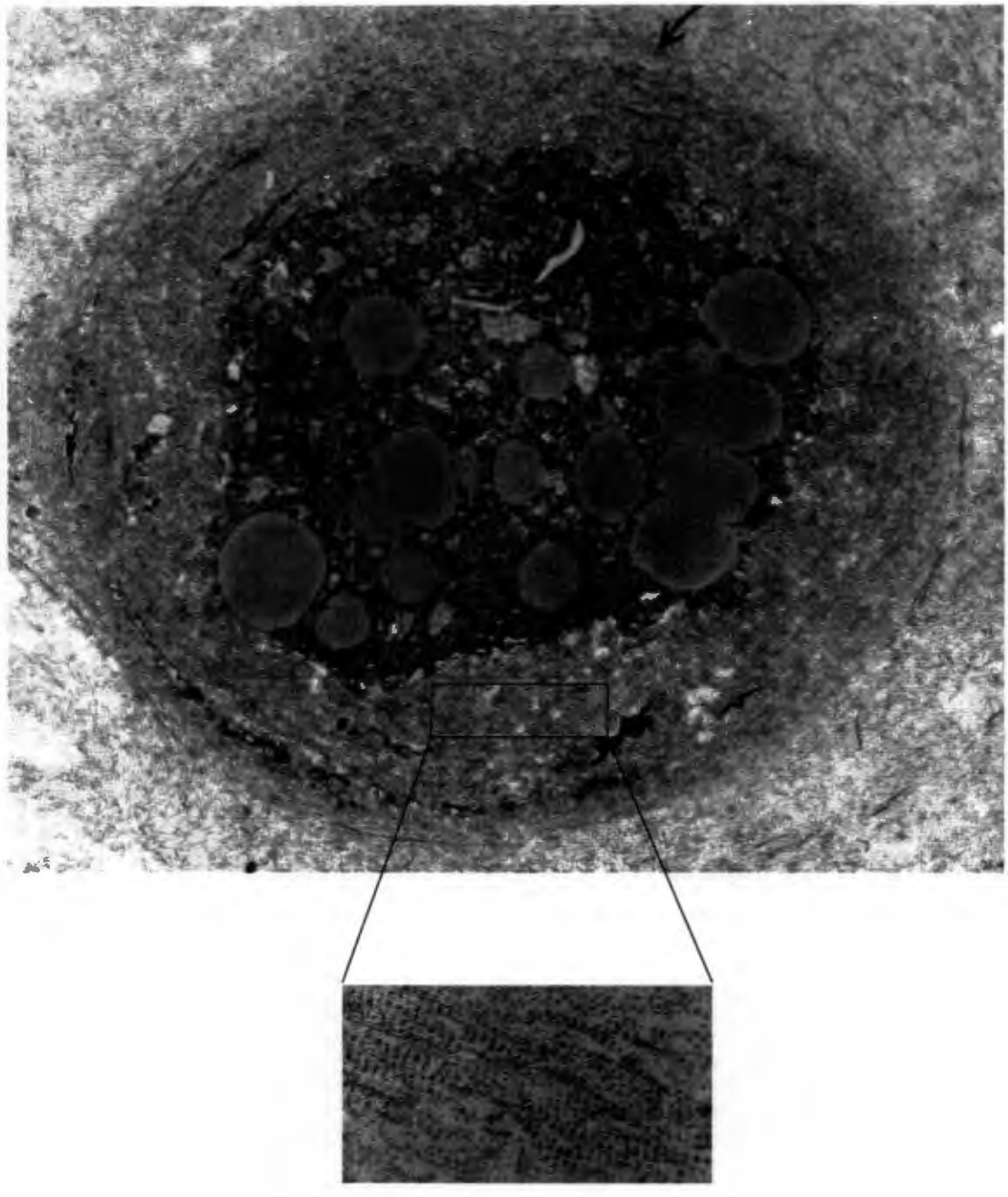

Fig. 7-1. An electronmicrograph of an articular cartilage specimen showing a chondrocyte surrounded by a broad banded aggregate which is typical of type VI collagen. The insert is a high power view of the type VI collagen aggregates.

A significant difference between the affected and control groups was observed with the COL9A1 marker, 509-12B1, due to a significantly higher number of homozygous individuals in the affected group (Table 7-I). Although the homozygosity displayed with this marker was not restricted to one specific allele and was observed for all the alleles that were present in the two study groups, allele 8 had the highest homozygosity frequency ($p<0.001$; $\chi^2 > 10.83$ at 1 degree of freedom [Emery, 1986]) (Table 7-II). Family studies to assess the significance of this increased homozygosity and whether there was an underlying molecular mechanism which resulted in the apparent 'loss of heterozygosity', could not be undertaken due to the unavailability of DNA specimens from the appropriate relatives.

Mutation screening of COL2A1

No variations were observed in the CSGE patterns of the affected individuals when compared to that of the normal controls.

7.4 DISCUSSION

The clinical features, natural history and family clustering of the SED-like hip dysplasia component of MJD could be explained on the basis of affected persons having a mild skeletal dysplasia, with a propensity to the development of severe and progressive degenerative OA. As the hip joints and the spine are most often affected, the condition could be regarded nosologically as a form of SED.

Table 7-I. The candidate gene markers which were tested and the frequency of the most common allele in 40 unrelated persons affected with MJD and in 30 controls. χ^2 was calculated at 1 degree of freedom. P=probability of linkage based on χ^2 .

GENE	[CA]n marker	NO. OF ALLELES	HETERO-ZYGOSITY FREQUENCY(%) Aff. : Con. (Lit.)	p	FREQUENCY OF MOST COMMON ALLELE Affected (%)	p	FREQUENCY OF MOST COMMON ALLELE Control (%)
COL2A1	VNTR	8	81 : 82 (73%)	>0.05	25	>0.05	26
COL6A2	VNTR	8	85 : 83 (70%)	>0.05	19	>0.05	22
	D21S1446	8	19 : 25 (69%)	>0.05	18	>0.05	22
COL6A3	D2S338	9	85 : 85 (78%)	>0.05	18	>0.05	28
COL9A1	509-12B1	8	36 : 65 (75%)	**<0.001**	25	>0.05	25
	509-8B2	10	80 : 79 (79%)	>0.05	26	>0.05	34
COL9A2	MYCL1	19	90 : 90 (87%)	>0.05	18	>0.05	12
COL9A3	D20S171	9	74 : 80 (63%)	>0.05	34	>0.05	22

GENE	[CA]n marker	NO. OF ALLELES	HETERO-ZYGOSITY FREQUENCY(%) Aff. : Con. (Lit.)	p	FREQUENCY OF MOST COMMON ALLELE Affected (%)	p	FREQUENCY OF MOST COMMON ALLELE Control (%)
COL10A1	D6S474	8	85 : 80 (64%)	>0.05	30	>0.05	32
	D6S1639	22	100 : 100 (91%)	>0.05	18	>0.05	18
	RFLP-HindIII	2	3 : 3 (50%)	>0.05	90	>0.05	90
COL11A1	D1S223	8	78 : 82 (74%)	>0.05	44	>0.05	36
	D1S248	10	81 : 74 (82%)	>0.05	29	>0.05	29
COL11A2	RFLP-MspI	2	20 : 20 (%)	>0.05	89	>0.05	85
	D6S291	7	85 : 93 (70%)	>0.05	33	>0.05	32
CRTL1	VNTR	13	72 : 75 (85%)	>0.05	19	>0.05	20
COMP	D19S215	7	60: 75 (34%)	>0.05	35	>0.05	33
	D19S222	6	75: 75 (67%)	>0.05	25	>0.05	33

GENE	[CA]n marker	NO. OF ALLELES	HETERO-ZYGOSITY FREQUENCY(%) Aff. : Con. (Lit.)	p	FREQUENCY OF MOST COMMON ALLELE Affected (%)	p	FREQUENCY OF MOST COMMON ALLELE Control (%)
DCN	VNTR	3	30 : 30 (32%)	>0.05	75	>0.05	82
CCAL2	D8S539	6	70 : 86 (58%)	>0.05	55	>0.05	50
	D8S565	6	73 : 68 (67%)	>0.05	40	>0.05	39
LUM	D1S95	7	65 : 50 (%)	>0.05	50	>0.05	53
FMOD	D1S306	8	88 : 94 (60%)	>0.05	20	>0.05	22
MMP3	D11S898	7	84 : 75 (79%)	>0.05	37	>0.05	30
CRTM	VNTR	4	60 : 60 (67%)	>0.05	42	>0.05	38
					48	>0.05	58

Table 7-II. The allele frequencies for marker 509-12B1 at the COL9A1 locus in the affected (40 individuals) and control group (30 individuals) from Mseleni. The homozygosity frequency for each allele is listed. χ^2 was calculated at 1 degree of freedom. Aff=affected; con=controls. P=probability based on χ^2.

	Allele ν (%) Aff:Con	**Observed Homozygosity Aff (%)**	**Observed Homozygosity Con (%)**	χ^2	**p**
1	5:3	3.6%	0.3%	10.58	<0.001
2	7:11	3.6%	0.5%	5.28	<0.05
3	25:25	14.3%	8.1%	1.30	>0.05
4	4:9	0	0.1%	0.04	>0.05
5	5:9	3.6%	0.5%	5.14	<0.05
6	21:18	10.7%	4.6%	2.28	<0.05
7	4:2	3.6%	0.1%	25	<0.001
8	27:21	25.0%	7.1%	12.50	<0.01

TOTAL HOMOZYGOSITY FREQUENCY:

Affected: 64.3% Controls: 36.4%

As previously mentioned, a similar disorder with the geographic appellation "Handigodu disease" (HJD) is present in a significant proportion of persons in two small, isolated caste groups of Karnataka, southern India [Agarwal et al., 1994, 1997]. The clinical manifestations of this autosomal dominant disorder resemble those of MJD and the issue of possible syndromic identity warrants consideration.

Early investigations into the causative factors of MJD focussed on the possibility that the disorder is the result of some unknown environmental factor. There are some parallels with Kashin-Beck disease, which occurs in Siberia which is reported to result from the ingestion of foodstuffs contaminated with aflatoxins [Nesterov, 1964]. Nevertheless, all attempts to identify a causative agent in the Mseleni environment have been inconclusive.

As MJD is confined to a specific population in a circumscribed area, genetic factors may be implicated. Indeed, the condition clusters in families, but there is no pattern of simple Mendelian inheritance. More complicated genetic mechanisms such as imprinting may be operative, but remain unproven. The situation is further complicated by the presence of a number of persons who have marked dwarfism, having a male mean height of 138.5cm and a female mean height of 136.2cm [Viljoen et al., 1993] in addition to the conventional osteoarthropathic manifestations. It may be supposed that these individuals are homozygous for the putative causative gene, but pedigree data do not support this concept.

Until the present study, no molecular genetic studies on MJD have been undertaken. The current investigations on ultrastructural analyses and genetic association was a

logistical extension of earlier genealogical studies of MJD [Lockitch, 1974].

HISTOLOGICAL ANALYSIS

Early investigations using transmission electron microscopy to determine the effect of OA on cartilage ultrastructure, provided evidence of damage to the collagen framework and an increase in water content in OA [Meachim et al., 1980]. In the present study, the accumulation of type VI collagen around the MJD chondrocytes suggests that type VI collagen is abnormal in cartilage in excised femoral head specimens [Ballo et al., 1996]. This phenomenon has, however, been seen in other forms of OA. Roncuzzi et al. [1990] provided evidence that chondrocyte metabolism and the collagen network undergo modifications in osteoarthritic cartilage. It is of interest that type VI collagen appears to be deposited abnormally within the cell [Shikata et al., 1993] and that mRNA expression is increased during wound healing [Oono et al., 1993], implying that type VI collagen expression may be increased in response to injury. The observation of excess type VI collagen around the chondrocytes in the present study was therefore considered to be a secondary generalised response in osteoarthropathies and not specific to the MJD condition.

HLA ANALYSIS

Certain non-Mendelian conditions such as ankylosing spondylitis, juvenile diabetes and psoriasis, amongst others [Kidd et al., 1977; Nair et al., 1997], have shown a significant association with the HLA complex, implying the existence of an underlying genetic defect in these conditions. In the present study, however, no association was found between HLA and MJD.

MOLECULAR ANALYSIS: COMPARISON OF ALLELE FREQUENCIES

The principle of allelic association and its potential for human genetics was first shown in the context of sickle cell anaemia [Kan and Dozy, 1978]. This powerful yet simple strategy involves the comparison of allele frequencies in a randomly selected group of affected individuals with those of an unaffected control group. The detection of non-random allelic association provides a high resolution approach for the precise mapping of a disease locus and has been applied successfully in the location of the genes for conditions such as cystic fibrosis [Kerem et al., 1989] and myotonic dystrophy [Harley et al., 1991], amongst others.

In the present study, no significant differences in allele frequencies were observed, between the affected and control groups, for markers from the COL2A1 locus which encodes the major component of articular cartilage and which is mutated in many familial forms of SED and OA [Ala-Kokko et al., 1990; Winterpacht et al., 1994; Ballo et al., 1998]. The absence of association between the COL2A1 markers and the MJD phenotype corroborates the mutation screening results, which did not detect a genetic defect. The allele frequencies of markers from the COL6A2 and COL6A3 loci were also investigated because of the type VI collagen aggregates around the chondrocytes which had been observed by electron microscopy. If there was a genetic modification of the type VI collagen genes which predisposed MJD affected individuals to the condition, it is reasonable to presume that a change in allele frequencies between MJD and normal controls would occur. No obvious differences in allele frequencies were, however, observed. The findings of the present study also ruled out the possibility of the other candidates, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, AGC1, DCN, FMOD, CRTL1, COMP, CCAL2, LUM,

CRTM and MMP3, being the major predisposing genes for MJD.

The COL9A1 gene cannot, however, be excluded as a candidate. The increased allele homozygosity which was observed in the affected individuals makes it a possible candidate for the MJD phenotype. These findings may be indicative of hemizygosity due to a null allele which renders the carrier susceptible to other genetic or environmental factors which may trigger the disorder. The significance of the apparent 'loss of heterozygosity' in COL9A1 therefore needs to be examined in nuclear families. Interestingly, genotypes using the other intragenic marker, 509-8B2, do not support an association between MJD and this gene suggesting that either complicated molecular mechanisms are operative in the MJD condition or that the homozygosity observed with 509-12B1 may be artifactual. However, the possibility of COL9A1 being a determinant gene for this osteoarthritic condition gets support from earlier findings by Nakata et al. [1993] and Fassler et al. [1994]. Those authors reported osteoarthritic-like changes in transgenic mice with non-inflammatory degenerative joint disease harbouring COL9A1 mutations. To date, no human chondrodystrophy has been associated with this gene. It is therefore necessary to follow-up this study with mutation screening of the COL9A1 gene and to pursue nuclear families in order to determine the significance of the apparent homozygosity.

In the event that COL9A1 becomes excluded as a candidate due to the absence of a mutation, then it would be necessary to embark upon a large scale study of the MJD population group, taking into account the advances which have been made in linkage and association studies. The method of investigation that was used in the present study was the most appropriate in the absence of relatives and the lack of affected

sibpairs. A comparison of allele frequencies is not as powerful as ASP analysis and family-based studies for revealing susceptibility loci and the existence of one or more predisposing mutations in a population. For future studies of this type, it would be necessary to revisit the Mseleni district for the purpose of collecting biological material the appropriate numbers of affected sibling pairs or from nuclear families, and then to pursue the molecular investigations for susceptibility loci using advanced statistical methodology such as sibpair analysis, and family-based association studies [Kruglyak and Lander, 1995; Thomson, 1995].

It may also be reasonable to conduct further studies of environmental factors in light of improved strategies and advances since the investigations of the early 1970's. Early investigations for toxins produced by the fungus of ground-nuts, *Aspergillus*, failed to implicate its toxin in the aetiology of MJD [du Toit, 1979]. The absence in Zululand of the fungus of bread grain, *Fusarium sporotrichiella*, which causes Kashin-Beck disease, excluded its toxic product as a possible causative factor. However, in the last decade, fumonisins, which are mycotoxins produced by the mold *Fusarium moniliforme*, were discovered. This mold is common on maize [Kommedahl and Windels, 1981, Marasas et al., 1984] and is apparently associated with increased bone and joint abnormalities [Sharby et al., 1973; Brown et al., 1992b]. Since the diet of the Mseleni population includes maize, it would be feasible to investigate the presence of the fumonisin toxin in affected and unaffected persons.

The pursuit of the gene(s) which are responsible for MJD is more than an academic interest. The condition is extremely debilitating in an already impoverished community. A knowledge of the susceptibility genes and the environmental factors which trigger MJD could have important implications for the lifestyle of this

community. Furthermore, it may provide valuable insights into the pathogenesis of the phenotypically similar HJD condition and also conventional age-related OA.

SECTION III

CHAPTER 8: CONCLUDING REMARKS

BIBLIOGRAPHY

APPENDIX

CHAPTER 8:
CONCLUDING REMARKS

Following the success in resolving the genetic defects in monogenic disorders, attention has now turned toward elucidating the genetics of complex traits. In the field of OA, major progress has been made in identifying the determinant genes which cause rare skeletal disorders in which OA is a secondary component. Molecular genetic strategies have led to the identification of alterations in the collagen genes and in the genes encoding non-collagenous components of the cartilage extracellular matrix. The COL2A1 gene has been implicated in the aetiology of numerous skeletal dysplasias, in particular the SEDs. Likewise, the genes encoding certain other components of the cartilage extracellular matrix have been identified as the determinants of a spectrum of skeletal dysplasias in which OA is a component.

The present study was focussed largely on monogenic skeletal dysplasias with OA as a secondary component, familial generalised OA without an underlying skeletal dysplasia and a chronic form of OA (MJD) which is present in a geographical isolate in southern Africa. The genetic bases of several monogenic chondrodystrophies were elucidated. Two clinically characterised SED entities, namely NSED and SEDCT, were linked to a common COL2A1 haplotype, and subsequently shown to carry a common disease-causing mutation in the gene. This larger unified group of SED patients will provide a means for acquiring

statistically significant data on disease aetiology, progression and modes of therapy.

In a family of Caucasian descent, a mutation in COL2A1 was found to underlie a Stickler-like syndrome. Analysis of data emerging from the current study with published literature was undertaken to determine whether genotype/phenotype correlations could be drawn on the type II collagenopathies. The present findings demonstrate that although most of the associated chondrodystrophies obey the phenotypic gradient of severity in type II collagen, phenotype/genotype correlations may be confounded by factors such as the position of the amino acid within the triplet repeat of the type II collagen helix (as seen in the South African family). For that matter, phenotypes cannot easily be deduced from the genotype or position of the mutation within the triple helical domain of COL2A1.

In a second family of Caucasian descent, a defective COMP gene was found to cause a mild Ribbing type MED. Previously, COMP mutations were shown to be causative of the severe Fairbank type MED and the allelic condition, PSACH. The present study expands the range of phenotypes which can be produced by COMP mutations and therefore suggests that this gene may also play a role in milder forms of OA.

A gene responsible for FOA of early onset may, in theory, be identified by linkage analysis in an affected kindred. Candidate genes may also be screened by mutational analysis in affected individuals. In this regard, Vikkula et al. [1989] reported linkage of the COL2A1 gene to OA in two large kindred. Mutational analysis of the gene did not, however, reveal the pathogenic mutation. It is

possible that their findings provided evidence of a susceptibility allele for the condition in those kindred rather than implicating COL2A1 as the determinant gene. Further candidate screening may reveal susceptibility alleles in other candidate genes, a combination of which is required to develop the condition. Other studies have demonstrated genetic heterogeneity in FOA by finding non-linkage to the COL2A1 gene [Meulenbelt et al., 1997]. Similarly, in the South African FOA family, no indication of linkage or association to one of the obvious candidate genes was observed, including COL2A1. It is also possible that the condition in this kindred is linked to an unknown bone or cartilage-expressed gene, and further investigation is warranted. It is also likely that the apparent Mendelian AD inheritance masks a slightly more complex inheritance pattern, which functions at least in a digenic manner (i.e. involving two, or even more, loci). In this instance using a greater number of 'affecteds only' in conjunction with candidate gene markers, could prove to be a powerful tool for identifying susceptibility alleles or predisposing polymorphisms.

The identification of genes whose products are responsible for the structural integrity of articular cartilage, provided support for our approach in investigating these and other tissue-specific candidate genes in a pilot-scale investigation of the chronic disorder, MJD. Association studies were our method of choice to locate the MJD gene(s). This approach has previously led to the localization of several susceptibility loci for complex disorders. The use of highly polymorphic markers linked to candidate loci was expected to produce statistically significant results, even in the absence of family structures. The current research was based on the presumption that MJD was homogeneous. For most loci, association between markers and the disease locus could be excluded. However, the significant

difference in heterozygote frequencies, which were observed between affected and control groups at the COL9A1 locus, warrants further investigation. As suggested in Ch. 7, a major molecular investigation of MJD needs to be undertaken, utilizing the appropriate sampling techniques which are required in accordance with more advanced strategies for linkage and association studies. Adequate sampling should also occur based on the premise that MJD is a complex disorder, and should facilitate non-parametric analyses for disease gene localisation.

The experimental findings reported in this thesis have contributed considerably to the elucidation of the molecular basis of several disorders in which degenerative OA is a feature. The lack of association between the wide range of obvious candidate articular cartilage genes and FOA and MJD, however, provides scope for further research, and may indicate that these latter disorders are aetiologically more closely related to generalised OA. A future extension of the current research is the investigation of non-familial generalised OA in which there is a collection of clinically confirmed patient material which is adequate for general genetic screening.

It seems prudent to conclude this thesis by saying that although establishing an association or linkage to a chromosomal locus is a crucial step in mapping a complex phenotype, the ultimate goal is to define the underlying mutation and to explore the functional mechanisms by which the disorder arises. With increasing health care costs and as the focus of medicine shifts to early detection and primary prevention, it becomes important to identify individuals who are at risk for common conditions. Moreover, insight into the causation of OA may have an immediate impact on the clinical management of affected individuals by suggesting novel therapeutic approaches. The ultimate long-term goal would be the prevention of this painful condition by clinical intervention at a presymtomatic stage, thereby alleviating the suffering and socioeconomic burden that the condition places on the population as a whole.

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APPENDIX

MATERIALS AND METHODS

1. DNA Extraction:

DNA was extracted from frozen whole blood using a modified version of the Genomix™ [*Talent*, Italy] kit. Essentially, red blood cells were removed, white blood cells were lysed and DNA was released. The DNA was then bound to an ionic exchange resin and all cellular debris was washed away. Thereafter, the purified DNA was eluted from the resin. DNA was checked for purity by reading the absorbance of the DNA solution and determining the A_{260}/A_{280} ratios. Digestion products of DNA samples which had been treated with restriction endonucleases were also examined.

1.1. Genomix protocol:

- 10ml frozen whole blood (collected in EDTA tubes) was thawed at 37°C, 2ml of Blood Washing solution (BWS) was added and the mixture centrifuged at 7000 rpm for 15mins to pellet out the nuclei and cell debris. The supernatant was removed and the pellet resuspended in 5ml of BWS. Centrifugation was repeated at 7000 rpm.
- The nuclei pellet was resuspended by vortexing in a mixture of 1ml of BWS and 1ml of water .
- Four ml of Lysing solution was added to the mixture which was then incubated at 68°C for 7mins.
- The lysate was transferred to a 14ml gel barrier tube and chloroform added to volume. The two phases were mixed by vigorously inverting the tube.

- The phases were separated by centrifugation at 5000 rpm at 10°C for 30mins. The upper aqueous phase was decanted into a 30ml glass tube [*Corex*, USA] which had been treated with diethyl pyrocarbonate [Maniatis et al., 1982].
- Eight ml of Precipitating solution was added to the aqueous phase and mixed by slow inversion until a filamentous DNA precipitate was visible.
- The liquid phase was removed the sediment resuspended in 4ml of Ionic Exchange solution, and the suspension left O/N at room temperature (RT).
- Eight ml of ethanol was added, followed by inversion,. to precipate out the DNA. The DNA was washed twice in 5ml of 70% ethanol, transferred to a 1.5µl microtest tube [*Eppendorf*, Germany] and centrifuged at 13 000rpm for 1min. All of the supernatant was removed and the DNA air-dried. The DNA pellet was finally resuspended in 200µl of sterile distilled deionised water (SDDW).

1.2. Determination of DNA concentration and purity:

The DNA concentration was calculated from the absorbance in water at 260nm (1 OD unit = 50µg/ml). The ratio of 260nm to 280nm absorbance values was used as a criterion of purity, a value greater than 1.5 being acceptable for DNA amplification by PCR.

2. POLYMORPHISM ANALYSIS

2.1. Microsatellite Polymorphism Analysis:

2.1.1. *DNA Primers*

Sequences for oligonucleotide primers for the amplification of di-, tri- or tetra-nucleotides were either selected off the Genethon map (http://www.cephb.fr/ceph-

genethon-map.html) or gleaned from scientific publications about the relevant gene. There were three ways of obtaining the primers viz:

(i) small aliquots were provided by generous overseas contacts,

(ii) the commercial company, Research Genetics, supplied them on order

(iii) the Department of Biochemistry, UCT, synthesized them on order

2.1.2. *DNA amplification for genotyping and haplotype analysis*

Amplification of DNA was performed in a total volume of 10ul containing 200ng genomic DNA; 0.5uM of each primer; 1X PCR buffer (50mM KCl/10mM Tris pH 8.4/ 1,5-2,5mM $MgCl_2$ or supplied by BioTaq^{TM}), 200uM of each of dATP, dCTP, dGTP, dTTP [*Boehringer Mannheim*, Germany]; 0.5units Taq Polymerase [(BioTaq^{TM}/) or (GibcoBRL,)]. Visualisation of polymorphic bands was achieved by the direct incorporation of 0.05ul (0.5μCi) of α-dCTP^{32} into each reaction. Each reaction mixture was overlaid with a drop of sterile liquid paraffin to prevent evaporation. Strict measures were taken throughout this and subsequent steps to ensure the safe use of radioactivity. All steps involving the handling of radioactive samples were conducted in an area which had been allocated for the use of radioactivity and the guidelines (compiled by the UCT Safety and Health committee) for safe usage were strictly adhered to.

Annealing temperatures for the different primer sets were determined by the following calculation:

4(G+C) + 2(A+T) - 5

Cycling conditions were as follows:

1 cycle of denaturation at 94°C/3mins

30-35 cycles comprising denaturation at 94°C/30secs, annealing at 50°C-65°C/30sec, extension at 72°C/45secs

1 cycle of extension at 72°C/5mins

10µl of loading dye (0.25% bromophenol blue/ 0.25% xylene cyanol FF/ 40% (w/v) sucrose) was added to each 10µl of PCR product.

2.1.3. *Resolution of length polymorphisms by PAGE*

Gel Matrix

A 6% denaturing polyacrylamide gel containing 15% urea, was made from a 40% stock solution of acrylogel 5, having an acrylamide to bisacrylamide ratio of 19:1 [*BDH*, UK]. Polymerisation was achieved by the addition of 10% ammonium persulphate (APS) [*BDH*, UK] and N'N'N'N'-tetramethylethylenediamine (TEMED) [*BDH*, UK].

Gel Size

Gels were 422cm long , 333cm wide and 0.35mm thick.

Electrophoresis Conditions

Plates were cleaned and treated firstly with ethanol followed by acetone before pouring the gel. Gels were allowed to set for 45mins or longer.

Samples were denatured at 98°C for 3-5mins and chilled on ice for 5-10mins before loading them onto the gel. 2-4µl of the denatured samples were loaded into each well and electrophoresed in 1XTBE at 50°C and at 70W constant power for 2-3hrs (depending on the expected fragment size) using a standard sequencing apparatus

[OMEG Scientific, South Africa). Gels were dried onto 3MM paper [Schleicher and Schuell, Germany] on a slabgel drier for 1-2hrs at 80°C and autoradiographed at -70°C for a period of 1-2nights.

10X TBE: 0.89M Tris/ 0.89M Boric Acid/ 0.02M EDTA pH8.0

2.2. Mutation Analysis:

2.2.1. SSCP

2.2.1.1. DNA amplification for SSCP analysis

Amplification of DNA was performed in a total volume of 10-20ul containing 200ng genomic DNA; 0.5uM of each primer; 1X KCL buffer (supplied by BioTaq), 200uM (dATP, dCTP, dGTP, dTTP); 0.5units Taq Polymerase (either BioTaq). The SSCP mobility shifts were visualised by silver staining the gels. PCR products were sized on 2% agarose gels and comparison with a 1kb MW marker [GibcoBRL, UK]. An equal volume of formamide loading dye (80% formamide/10mM EDTA pH8/1mg/ml xylene cyanol FF) was added.

2.2.1.2. Resolution of SSCP polymorphisms by MDE

Gel Matrix

A 0.5X MDE matrix (Hydrolink) either containing 10% glycerol, or without glycerol, and 0.6X TBE. Spacers and well-forming combs were 0.375mm thick.

Gel Size

Gels were 380cm long and 300cm wide and electrophoresed on a home-made system.

Electrophoresis Conditions

Samples were denatured at 98°C for 5mins and chilled on ice for 10mins before loading onto the gel. 10ul of the denatured samples were loaded into each well and electrophoresed in 0.6XTBE at R.T. at 2-10W (depending on the expected fragment size) for 16hrs.

Visualisation

Gels were stained over three stages:

20mins in 0.1% $AgNO_3$ solution

10-20mins in 1.5%NaOH/1.5%formaldehyde (until SSCP mobility patterns were visible) 5-10mins in 0.75% $NaCO_3$ (Often, it was impossible to remove the gel from the glassplates after staining. In that event, the gels were photocopied for permanent storage).

2.2.2. DNA Sequencing:

The Sequenase II (USB), and Sequitherm Excel I or Excel II (Epicentre Technologies) sequencing kits were used to determine the nucleotide sequences of exonic fragments. The manufacturers' protocol for direct incorporation of radioisotope was adhered to in both instances. The radioisotope of choice was α-$dCTP^{32}$.

Sequncing reactions were electrophoresed through 6% PAGE gels at a constant power of 70W. The duration of the run was determined by the length of the PCR fragment which was being investigated. The gels were dried on 80°C heated driers and autoradiographed at -70°C.

Protocol for Conformation Sensitive Gel Electrophoresis (CSGE)

Principle: CSGE is used to detect single-base mismatches in DNA heteroduplexes that contain one strand of wild-type and one strand of mutated DNA. With the mildly denaturing solvents--ethylene glycol and formamide--we can amplify the tendency of single-base mismatches to produce conformational changes such as bends in the double helix and thereby increase the differential migration of DNA heteroduplexes and homoduplexes during gel electrophoresis.

Specimen collection: ~4-8 µl of heteroduplexed PCR product from Genomic DNA.

Reagent	Store at	Additional information
polyacrylamide: BAP-solution	+4°C	**500 ml acrylamide** 40% w/v (Intermountain Scientific C-5550) **2.02 g BAP** (1,4-Bis[Acryolyl]Piperazine; Fluka 14470) **5 ml dH2O** Mix BAP with 5ml of dH2O, then add to acrylamid
Ethylene glycol	rt	Sigma E-9129
Formamide	-20°C	Gibco BRL 15515-026 store in single use aliquots (30ml).
20 x TTE	rt	**44.4 mM Tris-14.25 mM Taurine-0.1 mM EDTA buffer, pH 9.0** **432 g Tris** (Trizma Base, Sigma T1503) **144 g Taurine** (USB - 22072) **8 g EDTA** (Fisher - BP120-1) **Add dH_2O to 2000 ml** DO NOT AUTOCLAVE THE TTE BUFFER !
10% Ammonium Persulfate	+4°C	USB - 32810
TEMED	rt	Sigma - T-7024
10 X loading buffer	rt	**30ml Glycerol** (Glycerin Fisher G33-500) **.25 g Bromphenol Blue** (Fisher B-392) **.25 g Xylene cyanol FF** (Sigma X-2751) **Add H2O to 100 ml**
GeneMate or Acrylease	rt rt	Intermountain Scientific C-5990 Stratagene 300132
Ethidium Bromide		Sigma
Plates,spacers, comb		Inner Glass Plate (Kodak IB80520-size 37.5 x 43 cm) Outer Glass Plate (Kodak IB80540-size 37.5 x 45 cm) Gasket Kit (Kodak IB80600) Comb and spacer set (FMC 1mm, 37wells) Acco Binder Clips (size medium 5/8" capacity)

CSGE Gel:

Reagent	Final conc.	Volume for 1 gel	Volume for 2 gels
99:1 acrylamide 40%:BAP	15%	75 ml	150 ml
20 X TTE buffer	.5 X	5 ml	10 ml
dH_2O		68 ml	136 ml
Ethylene glycol	10%	20 ml	40 ml
Formamide	15%	30 ml	60 ml
10% Ammonium persulfate		2 ml	4 ml
TEMED		137 µl	274 µl

Procedure:

Enhance the heteroduplex formation by heating PCR products in the Perkin-Elmer Thermocycler as follows: Samples are heated at 98°C for 5 min then incubated at 68°C for 30 min. You can do this either as the last two cycles of PCR (easier...) or just before you put the samples on the CSGE gel. However, it is claimed that these steps increase the heteroduplex formation by only 5%, and I think that the most important function of these steps is to eliminate the appearance of the phantom bands.

Pouring the gel:

1. Clean glass plates with micro detergent, rinse well, then spray with 95% EtOH, and wipe each plate with a kimwipe. Coat the small plate with a small amount of Gene-Mate, let dry (or with Acrylease, coat every 5th-8th time.....).
2. Place spacers into long glass plate, cover with small plate and clamp.
3. Tilt the glass plates at a 45°C angle.
4. Gently pour the gel mixture between the two plates (if difficulties, use pipet or syringe).
5. Insert the 37 well comb between the two glass plates.
6. Clamp over the comb.
7. Let polymerize for one hour.

Running the gel:

1. After gel is polymerized, remove lower spacer and comb, attach plates to the Sequencing Rig.
2. Pour .5 X TTE Buffer into upper and lower chambers, check for leaks.
3. Pre-run for 10-30min 40 watts (optional).
4. Clean wells (with thin needle and syringe). Load Gel with appropriate amount (typically 3-6 µl) of PCR product mixed with 2-4 µl of loading dye.
5. Loading the gel in an asymmetric pattern helps to minimize chances of lane misidentification. Load 100ng of øx174 marker on one lane (optional).
6. Run about 8-9 h at 40 watts for 300-500 base fragments (for 45cm glass plate). For your convenience, I recommend to use a power supply with timer....If you experience problems (fuzzy bands etc.) try lowering the power.

Reading the gel

1. Remove plates from gel apparatus and separate small plate from large plate.
2. Stain gel with 200 µl concentrated ethidium bromide mixed with 2,000 ml tap H_2O for 10 min in large staining tray. Minimize background by not overstaining.
3. Visualize bands with a hand-held torch in dark room. If difficulties in finding the bands, transfer large enough piece of gel to blotting paper, and then visualize with UV torch. Note: Due to small amount of DNA and reflection from plate, bands may be hard to see by naked eye. Photography is critical to detection of band splits.
4. Cut a relevant portion of the gel with a scalpel, transfer with a piece of blotting paper and release onto the transilluminator by wetting with water.
5. Photography in this technique is used not only to document, but also to aid in detection of bands and doublet or triplets representing heteroduplexes of normal and mutant DNA regions. Bands must be photographed with a high quality camera and film apparatus. I recommend the use of high quality DCC camera with sufficient exposure time, brightness and contrast controls.
   Also, the Polaroid MP-4 camera and Polaroid type 57 film are effective, allowing careful focusing on close-ups of gel regions exhibiting bands. Multiple exposure times may be necessary to see bands of varying intensity in the same gel region. Photograph gel at varying apatures and exposures of varying times. Multiple short exposures may work better than one longer exposure to optimize band definition especially for bands that migrate close together.

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