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Molecular Genetic Approach to the study of Familial Dilated Cardiomyopathies

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Synopsis

In this thesis, I will report on the results of some molecular genetic studies carried out on patients with three inherited forms of Dilated Cardiomyopathy (DCM). DCM is defined as a primary disease of the myocardium associated with dilatation and impaired contraction of the left ventricle or both ventricles. Dilated cardiomyopathy represents a major cause of heart failure, arrhythmias, morbidity and mortality among cardiovascular diseases and it is the leading indication for heart transplantation. The occurrence of a familial form of dilated cardiomyopathy was very rarely reported in the past, but recent studies revealed a frequency of at least 20-25% of genetic transmission. Different modes of inheritance were recognized for the familial form: an autosomal dominant form, which is the most frequent form of familial dilated cardiomyopathy (AD-FDC); an autosomal recessive; an X-linked (XLDC), an autosomal dominant form with conduction defects and later development of DC (CDDC), and finally a newly recognized autosomal dominant form with clinical signs of myopathy (MDDC).

The population study was carried out on a total of 60 families for a total number of 255 evaluated individuals. Our attention was focused on three of these different forms of DCM.

Two families with suggestive X-linked mode of inheritance underwent linkage analysis which confirmed the association of the disease with the dystrophin gene. Further molecular analysis at the DMD gene level, allowed us to identify in one family a point mutation at the first muscular exon-intron boundary, abolishing the splicing of the muscular specific mRNA. In the second family, similarly to what occurs to Becker Muscular Dystrophy patients, a deletion of exons 48-49 was found by multiplex PCR analysis.

Among the different forms of DCM there is a newly identified form of FDC which shows additional signs of myopathy (MDDC). Since MDDC was recalling the better known XLDC form, determined by mutations of dystrophin, a candidate genes linkage analysis was performed for more than 30 loci where candidate genes coding for structural proteins of the cytoskeleton, the sarcolemma or the extra cellular matrix are localized. Unfortunately, this approach turned out to be unsuccessful.

The most frequent form of DCM is the autosomal dominant form of FDC (AD-FDC) which occurs in 55% of cases. Using a genome-scan approach, a first locus for AD-FDC was mapped in a large family pedigree (>120 examined

members) and confirmed in other two kindreds with AD-FDC. A cumulative lod score value of 3.69 was obtained with the polymorphic marker D9S153.

Subsequently, the candidate genes which map within the identified interval were analyzed. However, none was found in linkage with FDC. Since the candidate gene approach was unsuccessful, the map refinement of the region was carried out using new genetic markers which became available in the data banks. This map refinement led to the detection of new recombination events in critical patients, which dropped down the informativity of the identified FDC locus. Similarly, also analysis of the D9S156-D9S157 interval in chromosome 9, another locus with suggestive linkage at the first genome scan, did not produce further evidence for linkage.

These unconclusive results underline the problems that still hamper worldwide the identification of the genes responsible for FDC. These problems are related to the phenotypic complexity of the disease, its low penetrance, its genetic heterogeneity and the high chance of misleading clinical status definition. All these variables greatly limit the efficacy of a linkage analysis approach for this disease.

INTRODUCTION

DILATED CARDIOMYOPATHY

Cardiovascular diseases are the most important cause of death in developing countries (WHO, 1996). Among the wide variety of disorders affecting the cardiovascular system, there is a group of diseases characterized by an enlargement of the cardiac chambers, thinning of the ventricular wall, reduced contractility, heart failure and death (Keating and Sanguinetti, 1996). Such diseases are called dilated cardiomyopathies (DCMs).

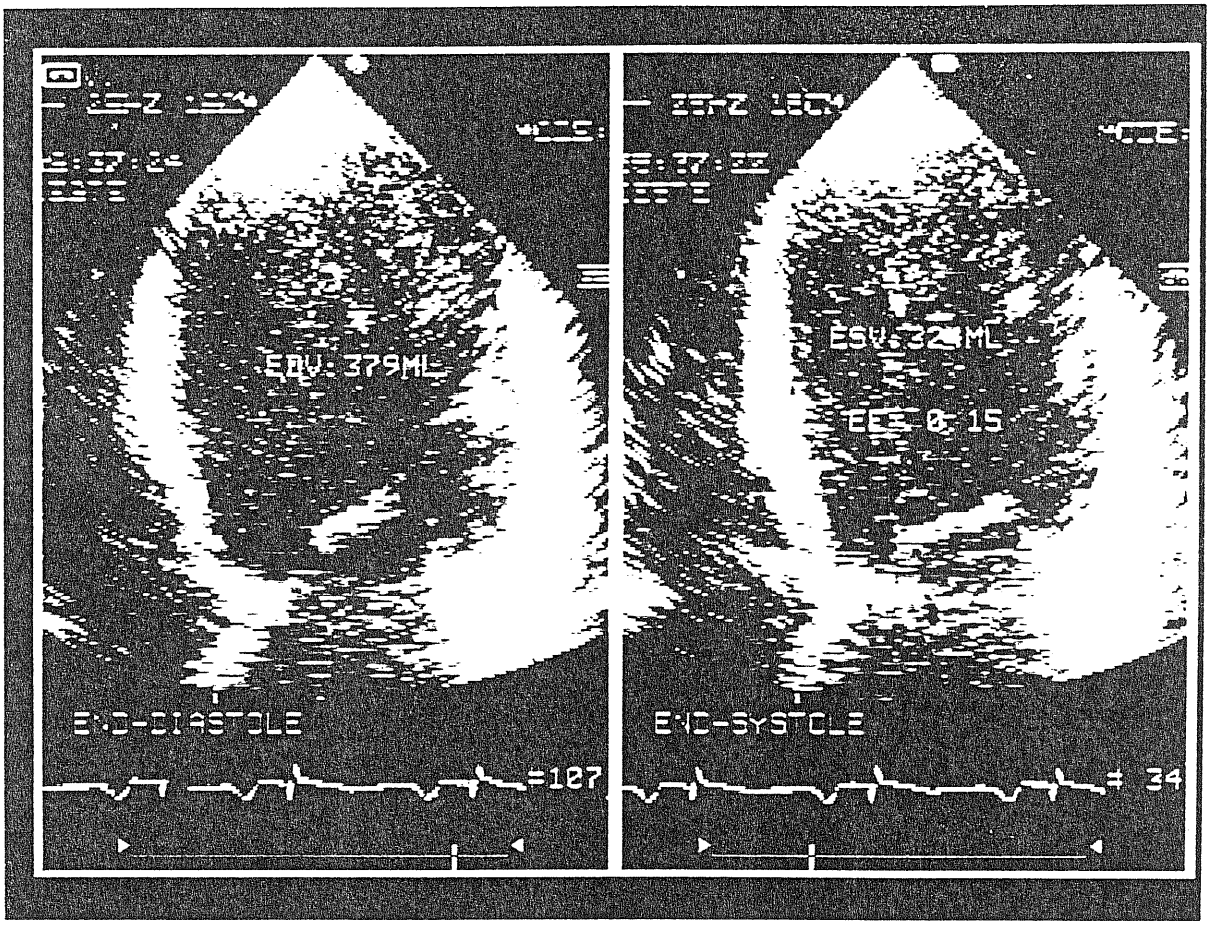
Dilated Cardiomyopathy (DCM) is defined as a disease of the myocardium associated with dilatation and impaired contraction of the left ventricle or both ventricles (WHO, 1996). DCM represents a major cause of heart failure, arrhytmias, morbidity and mortality among cardiovascular diseases and it is the leading indication for heart transplantation (Manolio *et al.*, 1992; Dec and Fuster, 1994) **(Fig. 1)**.

Clinical and social impact of dilated cardiomyopathy.

The clinical importance and the social impact of DCM are enormous. In fact, DCM is the primary indication for cardiac transplantation, with associated costs of about 200 million of USA dollars per year, which costs related to diagnosis and treatment of congestive heart failure secondary to DCM are several-fold higher (Manolio *et al.*, 1992). Furthermore, it is a fatal cause of heart failure characterized by ventricular dilatation and impaired systolic function (Manolio *et al.*, 1992). Nowadays, nearly 25,000 people die for cardiomyopathy in the United States every year (AHA, 1996).

Most cardiomyopathies are secondary, resulting from hypertension and valvular heart disease, but genetic factors are also clearly important. For

Figure 1. Echocardiography of the dilated heart of a FDC patient.



example, in the USA the prevalence of DCM is 37 cases per 100,000 people and it is estimated that genetic factors are key to the pathogenesis of the 20-25% of cases of idiopathic DCM (Michels *et al.*, 1992).

Role of genetic factors in the etiology of DCM.

In the last few decades, DCM has been largely investigated; nevertheless, at the moment, its etiology still represents a challenge. The diagnosis remains mainly based on the exclusion of any specific heart muscle disease, which means any myocardial disease associated with known cardiac or systemic disorders, such as ischemic, hypertensive, or valvular heart disease.

Due to our poor knowledge, the classification of the etiologic factors is still tentative (Cardiomyopathies, 1996): apart from the forms of DCM associated with excessive alcohol intake or with the exposition to other toxic agents, or from other cardiovascular diseases associated with or mimicking DCM, in a consistent group of patients, the etiology is far from being defined. In these cases, an autoimmune disease, a persistent viral infection and genetic factors are still considered to be the most probable causal factors, as in most of human diseases of unknown etiology. It is hypothesized that DCM represents the final common pathway of a heterogeneous group of disorders. Molecular genetic studies on specific cardiomyopathies, which demonstrate that both metabolic and structural defects of myocytes can lead to a common phenotype of myocardial dilatation and dysfunction, support this hypothesis.

The viral hypothesis.

The hypothesis of an inflammatory process of the myocardium due to a viral infection and subsequent viral persistence with chronic damage and development of heart failure has long been considered as one of the main causes of DCM (Kasper *et al.*, 1994). However, myocarditis, defined as a

pathologic process characterized by an inflammatory infiltrate of the myocardium with necrosis or degeneration (Aretz *et al.*, 1986), is indistinguishable from other inflammatory non-viral heart diseases, and remains in most cases of unknown origin.

Over the past decade, extensive investigative efforts have addressed the role of enteroviruses, in particular group B coxsackie viruses, in the pathogenesis of DCM and myocarditis using molecular biology techniques. However, in spite of the higher sensitivity and specificity of these techniques, the frequency of positive enteroviral genomes in myocardial tissue of patients with DCM was very variable, ranging from 0% to over 40% (Bowles *et al.*, 1989; Giacca *et al.*, 1994; Grasso *et al.*, 1992; Jin *et al.*, 1990; Severini *et al.*, 1993; Weiss *et al.*, 1992). Moreover, the relationship between clinical features and the presence of enteroviral genomes in the myocardium appears to be too variable, going from a significantly higher mortality (Why *et al.*, 1994) to a better prognosis compared to the enterovirus-negative patients (Figulla *et al.*, 1995). In our patient population, there were not significant differences in the clinical features between patients positive and negative for enteroviral genomes (Giacca *et al.*, 1994).

Whereas the pathogenetic role of enteroviral persistence in the chronic disease of the adults is questionable, in the pediatric population the role of a myocardial infection could be relevant. Viral genomes were found in 68% of myocardial samples of children (age 1 day to 19 years) with acute onset of left ventricular dilatation and dysfunction. However, other viruses were detected other than enteroviruses (30%), with a prevalence of adenoviruses (58%) followed by herpesvirus (8%) and cytomegalovirus (4%).

The autoimmune hypothesis.

Several abnormalities of cell-mediated immunity have been reported in DCM, such as a deficit in the function of T suppressor lymphocytes

(Eckstein *et al.*, 1982; Fowles *et al.*, 1979) and a reduced or absent NK activity (Anderson *et al.*, 1985; Maisch *et al.*, 1990)

It has been hypothesized that a lower T suppressor activity could lead to an altered production of autoantibodies by B lymphocytes in a subset of patients. Nevertheless, these alterations of the immune system are not cardiospecific and cannot be considered as a causal factor for the disease. On the other hand, considering the complexity of cell-mediated immunity, this aspect has not been sufficiently evaluated in DCM to draw any conclusion.

Humoral immunity has been more extensively studied. The presence of cardiac organ-specific autoantibodies has been reported, such as anti-cardiac mitochondria, anti-myosin, anti-actin, anti-myolemma, and, finally, anti-α and ß-myosin heavy chain, the last one characterized by a high specificity for the heart muscle and intercalated disks (Maisch *et al.*, 1990; Caforio*et al.*, 1992). Antibodies anti-ADP-ATP carrier of the mitochondrial membrane (Schulze *et al.*, 1990) were also described, which seem to interfere with the normal function of membrane calcium channels, and present some homologies with coxsackie virus RNA sequence. These antibodies could alter cardiac metabolism and function by calcium overload and by the reduction of the available ATP. Studies on anti-$ß_1$-receptor and anti-M2-muscarinic receptor antibodies suggest that these autoantibodies could have a functional role, in particular a positive chronotropic effect (Fu *et al.*, 1993; Magnusson *et al.*, 1994). As they are not strictly organ-specific, their significance in the pathogenesis of DCM is unclear.

The immune response is regulated by the major hystocompatibility complex (MHC). A major role of MHC in the pathogenesis of many autoimmune disease is largely accepted. Accordingly, an association between MHC class II antigens (in particular DR4) and DCM has also been hypothesized (Carlquist *et al.*, 1991). However, other studies on selected patient population with genetically determined DCM excluded any

association between MHC genes and the disease (Mestroni *et al.*, 1992; Olson *et al.*, 1995)

In spite of the large number of studies, the significance of immune activation in DCM is still unclear, and whether the described alterations represent a consequence or a causal factor has still to be established. Nowadays, clinical markers able to distinguish DCM patients with autoimmune activation are not known. Organ-specific cardiac autoantibodies appears to be associated with the early phase of the disease and with a better prognosis (Caforio *et al.*, 1990). However, follow-up studies are required to show if organ-specific autoantibodies could play a role in evaluating the stage and the prognosis of the disease.

The genetic hypothesis.

In the past, the presence of a familial aggregation of the disease was reported but certainly underestimated. After the first reports, the frequency of familial forms (familial dilated cardiomyopathy or FDC) was found to be only 2 % of patients based on a retrospective study carried out at the Mayo Clinic in 1981 (Fuster *et al.*, 1981). However, in the same institution, a subsequent retrospective analysis focused on the familiarity found a frequency of 6% of FDC (Michels *et al.*,, 1985). Analogous results were observed in our patient population first studied from 1979 to 1988 (Mestroni *et al.*, 1990): out of 165 patients prospectively studied, we observed that there was at least 1 relative with a documented cardiomyopathy in over 7% of our patients. The study was based on the observation of a known or suspected family history and further confirmation of FDC by means of a full evaluation of the affected family members.

However, it was a common opinion that these data underestimated the real incidence of the phenomenon, since the disease could either be latent, or clinically undetectable, or could not appear in the family history.

With the growing interest for this problem and the evolution of molecular genetic techniques, more recently, prospective controlled studies have been performed using a systematic screening of the families of DC patients irrespective of family history. With this approach, the occurrence of a genetic transmission was detectable in 20% - 30% of patients with DCM (Gregori *et al.*, 1996; Keeling *et al.*, 1995; Michels *et al.*, 1992), with a prevalent autosomal dominant trait **(Table 1)**. Even so, it could happen to underestimate the actual frequency of FDC due to limiting parameters as the possiblility of missing affected individuals, particularly when the pedigrees are small, and the reduced compliance of the families when the history of disease is not evident. Last but not least, the absence of early markers of disease, and the reduced penetrance (that is the proportion of carriers of the disease gene who manifest a clinical phenotype) render the detection of familiarity in FDC cases a difficult effort. Furthermore, the risk of disease was estimated to be approximately 20% in first degree relatives of DCM patients (Keeling *et al.*, 1995) and, in absence of a clinical examination, the risk of missing a familial trait is about 20% (Gregori *et al.*, 1996).

These findings had relevant implications: first, the relatives of DCM patients are at high risk of disease. This can be clinically not apparent, in particular in the young family members, due to the reduced and age-related penetrance of the disease (Mestroni *et al.*, 1994b).

The clinical and morphologic variability among FDC families suggested the existence of genetic heterogeneity, meaning that mutations in different genes could lead to the same phenotype. Moreover, different modes of inheritance were described. The pattern of transmission is more frequently autosomal dominant, suggesting a monogenic disorder. Less frequently, X-linked and recessive forms are observed. Finally, a polygenic inheritance, indicating the involvement of several disease or susceptibility genes, was also proposed (Zachara *et al.*, 1993). At least 5 different forms can be identified,

Study	N. of patients	% familial cases	Methods
Fuster et al, 1981	104	2	retrospective based on history
Michels et al, 1985	169	6	retrospective based on questionnairs
Mestroni et al, 1990	165	7	prospective, screening of suspected families
Michels et al, 1992	59	20	prospective, systematic family screening
Keeling et al, 1995	40	25	prospective, systematic family screening
Honda et al, 1995	117	25	systematic family screening
Trieste, 1997	100	48	randomization, systematic family screening (preliminary results)

Table 1. The estimated frequency of familial dilated cardiomyopathy during time and the methods odopted for the study.

based on their clinical and genetic characteristics: autosomal dominant FDC, autosomal recessive FDC, conduction defects with later development of dilated cardiomyopathy (CDDC), X-linked dilated cardiomyopathy (XLDC), dilated cardiomyopathy associated with muscle disease (MDDC) non-dystrophin related, and, possibly, mitochondrial DCM **(Table 2)**.

The development of molecular genetic techniques has provided the tools for the identification of the gene, or genes, causing the disease and the identification of the etio-pathogenetic mechanisms underlying the disease itself is considered now a research priority (Manolio *et al.*, 1992).

Molecular genetic progress in the study of primary myocardial diseases.

The recent application of molecular genetic tools to the study of some inherited forms of cardiovascular disorders, has allowed to get important insight into the molecular mechanisms underlying cardiac diseases. These studies have led to discover defects in several genes encoding for key role proteins to the cardiac function. Some of them encode for ion channels subunits as in the Long QT Syndrome (Keating *et al.*, 1991; Schott *et al.*, 1995; Sanguinetti *et al.*, 1995 ; Wang *et al.*, 1996 ; Keating and Sanguinetti, 1996]. Other genes encode for contractile proteins like β-Cardiac Myosin Heavy Chain, α-Tropomyosin, Troponin T and Cardiac Myosin Binding protein C, all causing the familial hypertrophic cardiomyopathy (Watkins *et al.*, 1995). Finally, recent studies have implicated a novel gene, G4.5, in the pathogenesis of X-linked DCM in Barth syndrome (Bione *et al.*, 1996).

Concerning DCM, biochemical aspects responsible for the disease remain still unknown (WHO, 1996). Thus, at present, the only possible research approach for this disorder is to directly achieve the gene or genes which are the cause of the disease, using a reverse genetics approach. Linkage analysis is the proper technical tool for this purpose.

DISEASE	TRANSMISSION	CHROM. LOCATION	GENE	FUNCTION
familial dilated cardiomyopathies				
"pure" FDC	autos.dominant	9q21-q22	unknown	
	autos.dominant	1q32	unknown	
	autos.dominant	10q21-q23	unknown	
	autos.recessive	unknown		
CDDC	autos.dominant	1p1-1q1	unknown	
	autos.dominant	3p22-p25	unknown	
XLDC	X-linked	Xq21	dystrophin	cytoskeleton
MDDC	autos.dominant	unknown		
mitochondrial cardiomyopathy	maternal trans.	mtDNA 3260	$tRNA^{LEU(UUR)}$	mtDNA transl.
	autos.dominant	mtDNA	multiple del.	respiratory chain
right ventricular cardiomyopathy				
ARVD1	autos.dominant	14q23-q24	unknown	
ARVD2	autos.dominant	1q42-q43	unknown	
ARVD3	autos.dominant	14q11-q22	unknown	
ARVD4	autos.dominant	2q31-q35	unknown	
unclassified cardiomyopathies				
DC and apical hypertrophy	autos.dominant	unknown		

Tab 2. List of the different DCM forms described so far and the genes or locus identified.

EPIDEMIOLOGICAL DATA OF FDC PATIENTS STUDY

Survey of genetic factors in dilated cardiomyopathy

Recent advances in the epidemiological data have been obtained from a clinical study carried out by the Cardiology Clinic of the University of Trieste which is our main collaborator for the DCM research.

An overall number of 70 families with familial cardiomyopathy was studied, and 372 family members were examined. Of them, 60 families with 281 individuals were considered as families with transmission of familial dilated cardiomyopathy (FDC): 96 individuals were affected, 144 healthy and 15 were considered with an "unknown status"; 26 were healthy acquired relatives; 13 affected individuals (20%) were under the age of 20 years.

In 39 families, the presence of 2 or more relatives with documented dilated cardiomyopathy led to the diagnosis of a familial form (Fig. 2). In 21 families, only the index patient was affected; therefore, the disease was considered "sporadic". Interestingly, the proportion of the observed generation showed a different distribution in the two groups. In FDC, the relationship with the index patient was: 1st degree in 20%, 2nd degree in 30% and 3rd or more in 50% of cases. In sporadic DC families, 77% were 1st degree relatives, 19% were 2nd degree and 4% were 3rd degree. These data suggest that FDC families underwent a more extensive screening, probably due to a greater compliance of the subjects. On the other hand, this may also determine an underestimation of the familial form due to a less extensive study of apparently sporadic DC families.

Analysis of the frequency of familial dilated cardiomyopathy

To obtain an estimation of the true frequency of the familial form of dilated cardiomyopathy, 100 out of 350 dilated cardiomyopathy index patients

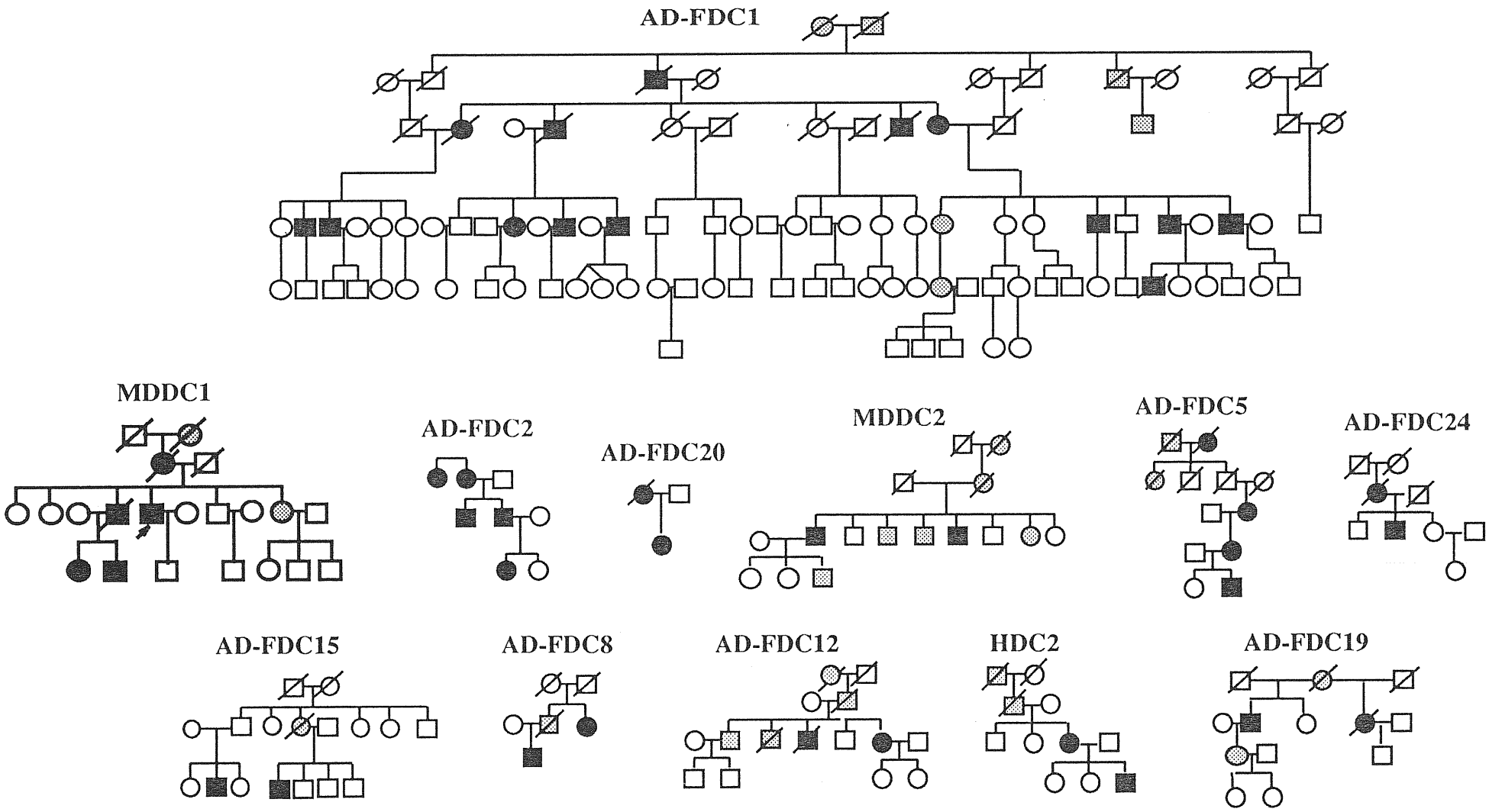


Figure 2. Pedigrees of families with familial dilated cardiomyopathy, consistent with autosomal dominant inheritance. Affected individuals are indicated by filled circles (females) or filled squares (males), whereas unaffected are indicated by open circles and squares. Individuals with equivocal or suspected DC are shown in gray.

were randomized. Of the 100 randomized cases, 72 were excluded from the analysis for the following reasons: 38 (53%) were excluded because dead or transplanted at the time of the study, to avoid bias or misclassification, 5 (7%) were lost to follow up, 18 (25%) refused the family screening because living too far and 11 (15%) for personal reasons. There was a family history of dilated cardiomyopathy in 4 excluded cases (5.5%), all in the group of deceased patients, while 68 patients were apparently "sporadic" DC. There were no significant differences between the index cases included and excluded from the analysis.

Of the 28 cases (25 families) included in the analysis 155 relatives were clinically evaluated (28 affected and 127 unaffected), belonging to 2 to 6 generations kindreds. There was a documented family history of dilated cardiomyopathy in 12 independently observed cases, belonging to 10 families. In other 13 patients, a sporadic condition was confirmed; however, in 2 of them, there was a history of suspected sudden deaths in the family, that were not verifiable. Finally, 3 cases believed to have a sporadic form were found to be familial. These data led to a crude estimate of the frequency of familial dilated cardiomyopathy of 48% **(see Tab. 1)**.

Characterization and classification of FDC

FDC families represent a genetically heterogeneous group. A novel classification of familial cardiomyopathies can be characterized by dilatation and left ventricular dysfunction, based on the proposed clinical features, different patterns of genetic transmission and, when available, on the molecular genetic data, presented in **Table 3**. According to this classification, distinct forms of FDC are identified. Among them, there is a newly identified form of dilated cardiomyopathy with subclinical skeletal muscle disease, named MDDC. MDDC recalls the XLDC clinical features, but shows an autosomal dominant pattern of trasmission (Mestroni et al., data not

	AD-FDC	AR-FDC	XLDC	MDDC	H-DC	RP-DC	CDDC
Genetic and molecular data							
chromosome	9 [q13-q22] () 1 [q32 ()]	-	X [q21] ()	-	-	-	1 [q1-q1] () 3 [p22-p25] ()
gene	unknown	-	dystrophin	unknoiwn	-	-	unknown
Present study: main features							
n. kindreds (%)	21(56%)	6(16)	4(10)	3 (8)	3 (8)		1(3%)
n. subjects studied (affected)	132(50)	16(10)	6(4)	22(7)	10(7)		2(100)
age at diagnosis (range)	43 (4-82)	21(7-53)	29(19-39)	34(16 46)	34 (18-46)		40
gender	3:2	4:1	1:0	6:1	1:1		1:0
LVEDD	6.6 1.5	6.3 0.9	6.7 1.8	6.5 1.4	5.6 1.0		-
LVEF	34 11	31 12	32 24	28 12	39 16		-
Mildly dilated (%)	42	20	50	57	28		-
Conduction defects (%)	16	20	0	86	0		100
VT	72	43	50	83	14		100
CK	0	0	75	71	0		-
organospecific antibodies	39	33	0	1	0		-
Morphometry							
N. patients studied by morphometry	26	6	4	5	2	1	0
myocellular area (μ^2)	1.9 ± 0.8	2.2 ± 0.6	1.6 ± 0.7	1.7 ± 0.6	1.8± 0.4	2.0 ± 0.8	-
nuclear area (μ^2)	0.1 ± 0.1	0.2 ± 0.04	0.1 ± 0.05	0.1 ± 0.05	0.1 ± 0.1	0.2 ± 0.01	-
myofibrillar area (μ^2)	1.1 ± 0.6	0.9 ± 0.5	1.0 ± 0.52	1.5 ± 0.8	1.0 ± 0.3	0.8 ±0.5	-
collagen volume fraction (%)	13.8 ± 9.7	6.6 ± 5.4	17.3 ± 15.0	13.8 ± 11.6	7.4 ± 3.5	-	-
adipose tissue (%)	6.5	-	-	-	-	-	-

Table 3. Summary of molecular genetics, clinical, immunological and morphometric findings in familial dilated cardiomyopathy

Legend: AD-FDC: autosomal dominant dilated cardiomyopathy; AR-FDC: autosomal recessive DC; XLDC: X-linked DC; MDDC: autosomal dominant DC
with skeletal muscle involvement; H-DC: DC with apical hypertrophy; RP-DC: DC associated with retinitis pigmentosa; CDDC: conduction defect with dilated
cardiomyopathy. The morphometric data refer to the mean value derived from at least 3 different samples for each patient.

published). Although it represents a not very frequent FDC form, according with the study of the University of Trieste, it increases the already wide complexity of the clinical scenario of DCM. One of these MDDC family was used for the molecular genetic studies (see later on this thesis).

MOLECULAR GENETIC STUDIES OF FDC

After the introduction of molecular genetics in the study of cardiomyopathies, important progresses have been made towards the identification of the genes causing the disease: five disease loci have been mapped so far, which segregate with the autosomal dominant forms of dilated cardiomyopathy **(Fig. 3)**. Furthermore, dystrophin has been demonstrated to cause the X-linked form of the disease. The following paragraphs will focus on the clinical and molecular genetic findings in the study of inherited DCM and on the future perspectives.

Autosomal dominant familial dilated cardiomyopathy.

Autosomal dominant FDC represents the most frequent form of inherited DCM, characterized by development of chamber dilation and systolic dysfunction usually in the second to third decade of life, with progressive heart failure and ventricular arrhythmias. This group represents the majority of families in the patients study of the University of Trieste (22 kindred, 56% of cases). As far as the putative disease gene (or genes) is concerned, this is still unknown. The low penetrance of the disease, the absence of early clinical markers and the premature mortality have always been obstacles in collecting families with an adequate sample for molecular genetic studies.

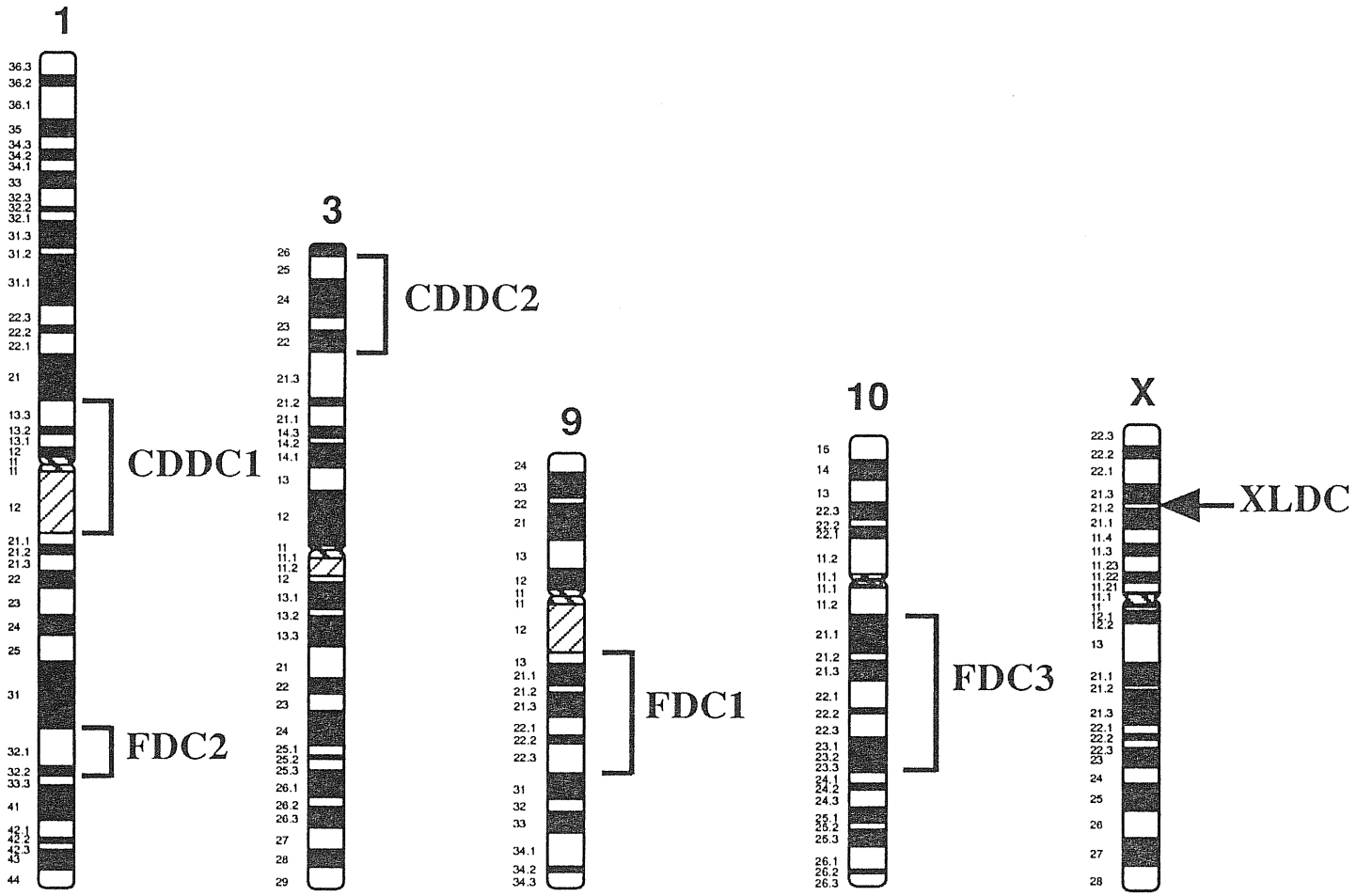


Figure 3. Diagrams of the four chromosomes containing known disease gene loci for different forms of DCM. The disease loci, identified by square brackets, were mapped on chromosome 1q1-p1 for the CDDC locus [Kass et al., 1994], 1q31-q32 for FDC2 locus [Durand et al., 1995], on chromosome 3p22-p25 a CDDC2 locus [Olson et al., 1996], on chromosome 9q13-q22 the FDC1 locus [Krajinovic et al., 1995] (see later this thesis), on chromosome 10q21-q23 the FDC3 locus [Bowles et al., 1996], and chromosome Xp21 with the dystrophin gene (DMD), cause of XLDC.

First, linkage of FDC with candidate genes was tested. Candidate were considered genes encoding contractile proteins, proteins involved in the metabolic pathways, and, according to the autoimmune pathogenetic hypothesis, genes involved in immune regulation. However, none of them was found in linkage with FDC (Krajinovic *et al.*, 1994; Mestroni *et al.*, 1994a; Olson *et al.*, 1995). Instead, using a whole-genome random screening approach, a first locus for autosomal dominant FDC was mapped to chromosome 9 in a large Italian kindred and in other two smaller families (see later this thesis). A second locus has been localized on chromosome 1q32 in large Utah family (Durand *et al.*, 1995). Several candidate genes coding for cytoskeletal and regulatory proteins, and proteins involved in metabolic pathways map to these chromosomal regions. However, the putative disease genes are still unknown.

Another locus for autosomal dominant FDC has recently been reported on the short arm of chromosome 10 (10q21-23) (Bowles *et al.*, 1996) in one large kindred. Interestingly, this form of DCM is characterized by the association with mitral valve prolapse and by a high penetrance, further confirming the heterogeneity of this disease. Because of the morphological features of this form, muscle cytoskeletal proteins, such as vinculin, laminin, ankyrin and actin, are considered as putative candidate genes.

Familial dilated cardiomyopathies with sub clinical muscle involvement.

X-linked dilated cardiomyopathy (XLDC) is familial heart disease presenting in young males as a rapidly progressive congestive heart failure, without overt clinical signs of skeletal myopathy (Berko and Swift, 1987). The affected family members can have a mild increase of muscle creatin kinase MM-CK) despite clinically normal muscle strength. Heterozygous females tend to present later in life with a slower progression to heart failure (over a decade or more). From the clinical standpoint, the disease in the affected does

not differ from that found in other forms of familial dilated cardiomyopathy with different patterns of inheritance.

Recently, it has been demonstrated that specific dystrophin defects are responsible for this form of dilated cardiomyopathy **(Fig. 4)**. Dystrophin is a very large rod-shaped protein of 472 kDa, encoded by the largest gene of the human genome (79 exons spanning 2,500 kb). The studies of dystrophin gene expression established that it is present in neurons, smooth muscle, cardiac and skeletal muscle (Ahn and Kunkel, 1993). Mutations or deletions of this gene cause the Duchenne and Becker muscular dystrophies. In one study (Nigro *et al.*, 1990), it was reported that more than one third patient with Duchenne muscular dystrophy develop signs of cardiac dysfunction in early teens and virtually all of them had dilated cardiomyopathy in their late teens. Moreover, in patients with Becker muscular dystrophy, a high incidence (60-72%) of myocardial involvement was observed. In the cases with mild skeletal muscle involvement, cardiomyopathy may be the primary clinical feature and can be rapidly progressive, leading to transplantation or death within 1 or 2 years after the initial presentation (Melacini *et al.*, 1996).

In 1993, three studies demonstrated that a dystrophin gene defect was the cause of XLDC. Towbin and collaborators (Towbin *et al.*, 1993) found linkage of two families showing X-linked transmission of the disease (one of these was the first XLDC family originally described by Berko and Swift (Berko and Swift, 1987) with the dystrophin locus. In 3 other families, (Muntoni *et al.*, 1993; Yoshida *et al.*, 1993) dystrophin gene deletions affecting the region of the muscle promoter and of the exon 1 of the muscle isoform were found, suggesting a critical role of the 5' end of the gene for the expression of dystrophin in the heart. More recently, two different point mutations were identified in XLDC families. The first mutation (Milasin *et al.*, 1996), a G -> T substitution, alters the consensus sequence of the splicing site at the first

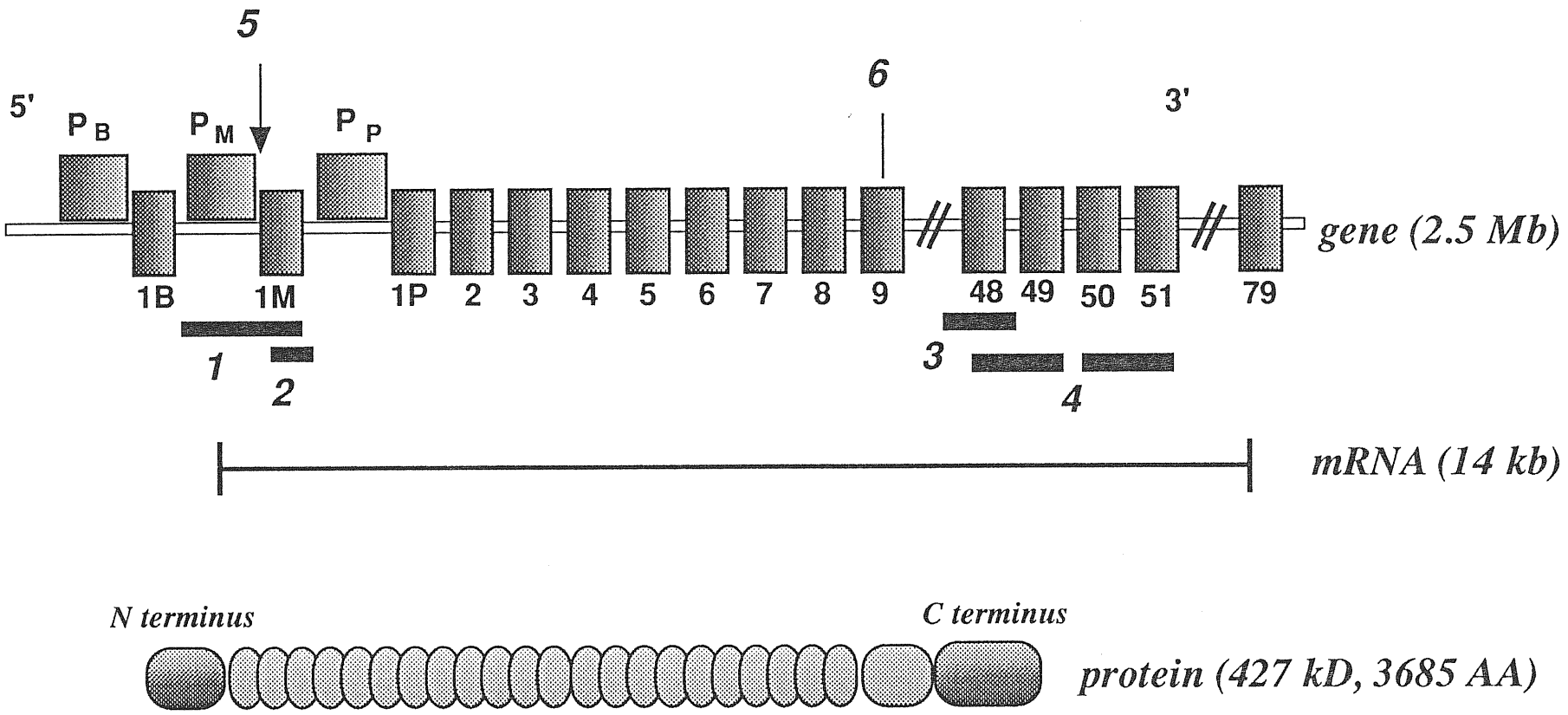


Figure 4. Structure of the dystrophin gene regulatory elements which drive the transcription of the Brain, Muscle and Purkinje mRNA isoforms.Under the dystrophin gene structure, are indicated the deletions found to cause the XLDC phenotype (numbered from 1 to 4). These deletions affect the muscle and brain promoter region and the exon 48 to 51 region as well.

muscle exon-intron junction and abolishes the production of the muscle isoform of the gene. This mutation is described in this thesis.

A second point mutation has been recently identified in exon 9 (Ortiz-Lopez *et al.*, 1997), a missense mutation A -> G resulting in the substitution of the amino acid threonine to alanine at position 279 (T279A) of the protein.

Several potential mechanisms can be hypothesized to explain the cardiospecificity of these dystrophin mutations, such as differences in function between cardiac and skeletal muscle dystrophin (Beggs, 1997).

In 1996 Meng and collaborators, demonstrated an association of dystrophin with the Z-disc region of cardiac muscle but not with analogous Z-lines of skeletal muscle. Moreover, dystrophin localizes to the transverses tubules of cardiac but not skeletal muscle. These localizations are mediated by tissue specific bindings to other protein components of the relevant structures, hence, an amino acid substitution at a critical site could alter such an interaction. Finally, different defects within critical functional regions of the dystrophin protein, or defects in the regulation of the expression of the dystrophin mRNA could lead to the phenotype of XLDC.

By analogy with dystrophin, other cytoskeletal proteins appear to be potential candidates for causing dilated cardiomyopathy. In 1996, Fadic and collaborators described the deficiency of alpha-sarcoglycan (adhalin), one of the DAG-complex proteins, in one patient with DCM associated with signs of muscle dystrophy (Fadic *et al.*, 1996). The absence of transcription of metavinculin (the cardiac isoform of vinculin) has been shown in one patient with family history of DCM (Maeda *et al.*, 1997). In 1997, Nigro et al., showed that the gene coding for δ-sarcoglycan was responsible for the autosomal recessive Syrian hamster (BIO14.6) cardiomyopathy.

Finally, families with matrilineal transmission of the disease suggest mitochondrial DNA (mtDNA) defects. This disease can be difficult to identify,

due to the variability of heteroplasmy and the increase of deletions with the advancing age, and is usually a multisystem disease. DCM can be found in MELAS syndrome, MERFF syndrome, NADH-coenzyme Q reductase deficiency, Kearns-Sayre syndrome and MIMyCa (Schowengerdt and Towbin, 1995; Zeviani *et al.*, 1991). Multiple deletions of mtDNA have been described in FDC (Suomalainen *et al.*, 1992), as well as in sporadic DCM patients (Remes *et al.*, 1994), but their causal role has not yet been demonstrated.

Conduction system disease and dilated cardiomyopathy.

A peculiar and rare form of familial DCM is a dominant cardiac conduction system disease with later development of dilated cardiomyopathy (CDDC) (Graber *et al.*, 1986; Mestroni *et al.*, 1990). The affected family members manifest arrhythmias and atrio-ventricular block in the second to third decade of life, and a progressive cardiomegaly and heart failure in the fifth to sixth decade.

A first linkage study carried out in a large Ohio family mapped the CDDC disease gene in the centromere of chromosome 1 (1p1-1q1) (Kass *et al.*, 1994). Recently, in another CDDC family of Swiss-German ancestry linkage was found with the short arm of chromosome 3 (3p22-p25) (Olson and Keating, 1996). Also in this case, the disease genes are still unknown.

Constrains of the molecular genetic study of FDC

Since the knowledge about the biochemistry of DCM is very poor, the compulsory way to follow, is still to pursue a reverse genetic approach which takes advantage of a powerful tool of molecular genetics as the linkage analysis.

Any progress in the molecular characterization of the DCM will be remarkable due to the extreme difficulty in recruiting a critical number of patients and collecting suitable families for the molecular genetic study.

Furthermore, the relative small size of human pedigrees, obliges the researcher to find out new technologies or to pool different laboratories in order to create a enough large register of families and single patients which will enable to bypass those problems. DCM is a very difficult disease to study also because its potentially pluricausal nature and the fact that it is often a diagnosis of exclusion.

Another important clinical feature which makes genetic studies very tough is the problem of *penetrance*. The penetrance is the probability that a genotype will yield the predicted phenotype.

FDC has an age-related and incomplete penetrance which could lead to false negative clinical diagnosis **(Fig. 5)**. Standing this, the setting of feasible diagnosis criteria which should be clear and easy to follow, is strongly required.

An issue which complicates the use of large sets of small families is the presence of genetic heterogeneity. In fact, several diseases or other phenotypes are said to be heterogeneous when more than one gene is involved as the cause of the disease.

In fact, studies on systemic diseases associated with dilated cardiomyopahty indicate that genes encoding for proteins with different functions can lead to the same phenotype of myocardial dilatation and dysfunction (Dec and Fuster, 1994; Kelly and Strauss, 1994).

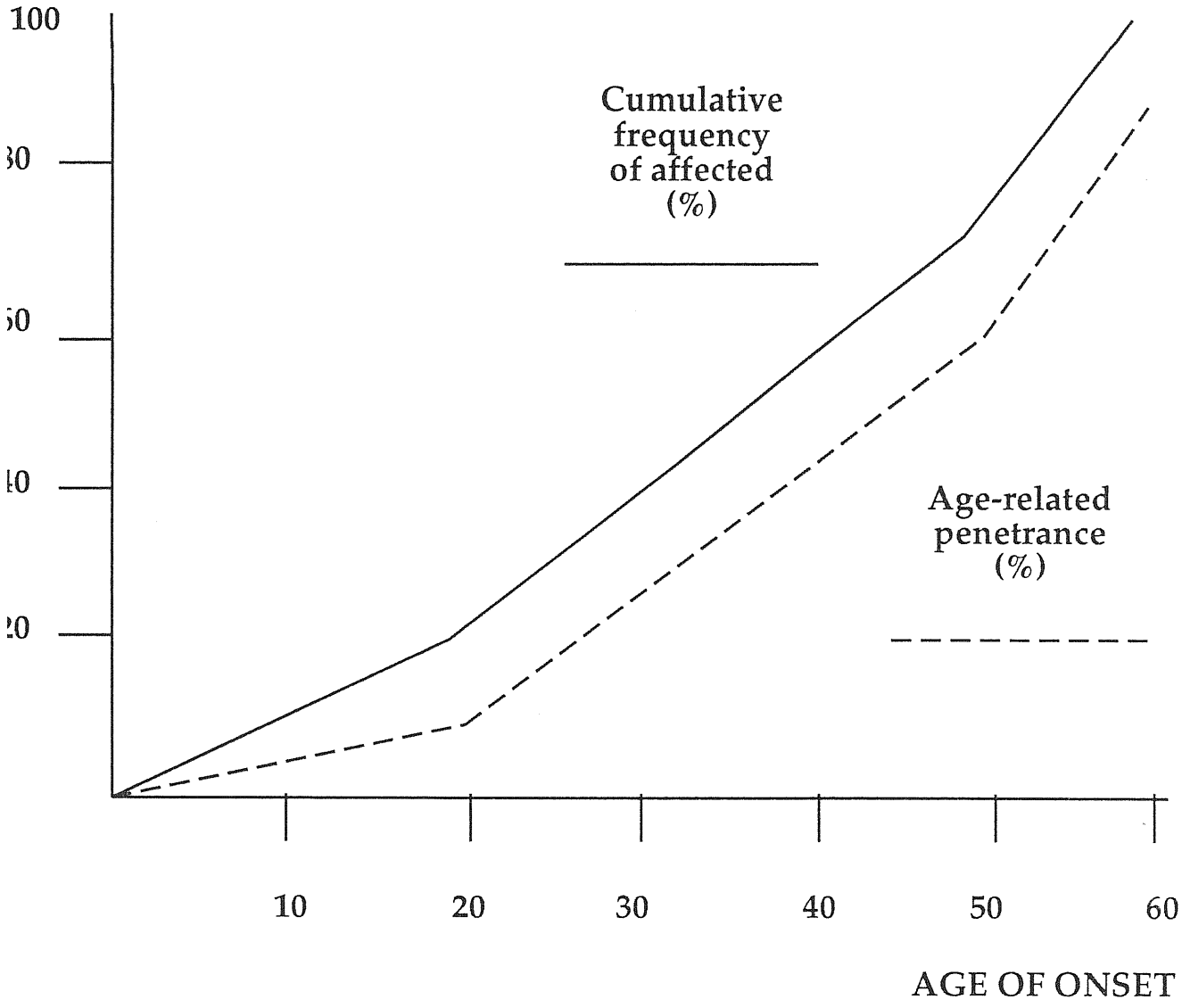


Figure 5. Penetrance of familial dilated cardiomyopathy

PATIENTS AND METHODS

PHENOTYPIC CHARACTERIZATION OF PATIENTS

Diagnostic criteria

The diagnosis of dilated cardiomyopathy was made according to the statement of WHO/ISFC (Brandeburg *et al.*, 1981) and to the guidelines of the National Heart, Lung, and Blood Institute Workshop on the Prevalence and the Etiology of Idiopathic Dilated Cardiomyopathy (Manolio *et al.*, 1992). As previously described (Mestroni *et al.*, 1994b), family members were evaluated by history, physical examination, electrocardiography, as well as by M-mode, cross sectional and Doppler echocardiography, interpreted by two independent observers. Normal values for echocardiographic measurements were determined according to standard protocols (American Society of Echocardiography 1989; Henry *et al.*, 1980). Diagnosis of dilated cardiomyopathy was based on the presence of fractional shortening (FS) < 25% (> 2D) and/or ejection fraction < 45% (> 2D) in the presence of a LVEDD > 117% [> 2D of the predicted value of 112% corrected for the age and BSA (Henry *et al.*, 1980)], excluding any known cause of myocardial disease. Exclusion criteria were: blood pressure > 160/110 mmHg documented and confirmed at repeated measurement, obstruction > 50% of a major brach of coronary artery, alcohol intake > 100g/die, persistent high rate superventricular arrhytmias, systemic diseases, pericardial diseases, congenital heart disease and cor-pulmonale (WHO, 1996). Idiopathic myocarditis was not excluded.

Relatives showing signs of cardiomyopathy underwent, whenever possible, ventriculography, coronary angiography, endomyocardial biopsy, and a comprehensive arrhythmologic evaluation (Holter, signal average

electrocardiogram, exercise test). In the remainder, the evaluation was completed by non-invasive techniques and review of previous hospital records. For the deceased relatives, hospital records were examined when available, family physicians were interviewed, and multiple informants among close relatives were consulted for accuracy of diagnosis.

Hystology and immunocytochemistry

A needle biopsy of skeletal muscle and left ventricular endomyocardial biopsy were obtained from all available individuals, after informed consent. The samples were studied according to standard histological procedures as: haematoxylin and eosin, Gomori's trichrome, periodic acid Schiff, cytochrome oxidase, oil red O staining (Dubowitz V., 1985). Moreover, immunocytochemical examinations were performed using a biotin-streptavidin Texas-red method.

Six micron unfixed cryostat sections were immunostained using a panel of antibodies to all the muscle cell compartments (extracellular matrix, sarcolemma, sub-sarcolemma and cytoskeleton) using antibodies against dystrophin (N-ter, C-ter and mid rod domain), desmin, β-dystroglycan (43 DAG), α-sarcoglycan (50 DAG), β-1-laminin and fetal myosin (Novocastra Laboratories, Newcastle-upon Tyne, UK), as described (Muntoni *et al.*, 1993).

GENETIC STUDIES

DNA extraction from whole blood samples and lymphocytes

DNA purification from whole blood samples was performed by the salting out procedure (Miller *et al.*, 1988). Five ml of blood in 10 mM EDTA pH 8.0 were transferred to 50 ml Falcon tubes and 45 ml of Lysis Buffer (0.32 sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ and 1% Triton X-100) at 4°C

were added. After gentle mixing, the tubes were left on ice for 10-15 min. The tubes were then centrifuged for 10 min at 3000 rpm at 4°C. By this centrifutation, clear pellets became visible and, after discharging the supernatants, they were washed 2-3 times with Fisio Buffer (0.075 mM NaCl, 0.025 M EDTA pH 8.0) by centrifuging as described above.

After washing, pellets were resuspended in 3 ml of Buffer A (10 mM Tris-HCl pH 8.0, 400 mM NaCl, 2 mM EDTA) to which 200 µl of 10% SDS and 50 µl of Proteinase K (10 mg/ml in 1% SDS, 2 mM EDTA; Sigma, S.louis, MI, USA) were added. These reactions were incubated at 37°C overnight. After incubation, 1ml of a saturated NaCl solution (approximately 6 M) was added and the tubes were shaked vigorously for 15 sec. By this treatment, the precipitated protein pellet upon centrifugation at 3000 rpm for 15 min. The supernatants were then transferred to 15 ml centrifuge tubes, taking care not to touch the white floating precipitate. A further centrifugation was then carried out at 3000 rpm at room temperature. The supernatants were then transferred to 50 ml tubes and 1 volume of isopropanol (kept at room temperature) was added. The precipitated DNA was removed by a hooked pasteur pipette and dipped into 1 ml of 70% ethanol for 30 sec. DNA was then resuspended in 500 µl of TE (Tris 10 mM; EDTA 0.1 mM) by gentle agitation overnight at room temperature.

When DNA extraction had to be performed from isolated lymphocytes or lymphoblastoid cell lines, cellular pellets were resuspended in 3 ml of Buffer A and the same procedure as above was followed.

Separation of PBMCs

Ten ml of heparinized blood samples were processed within 24 hours from withdrawal. They were mixed with 10 ml of RPMI 1640 (Gibco BRL Life Technologies; Paisley, Scotland) and then layered over 8 ml Ficoll Hypaque (Sigma) and centrifuged for 20 min at 1500 g at room temperature. The

peripheral blood mononuclear cells (PBMCs) ring was recovered and washed three times with RPMI 1640. Cells were either immediatly immortalized by EBV infection or frozen in a 10% DMSO and 90% of fetal calf serum solution to preserve vitality.

Establishment of B-lymphoblastoid cell lines

The EBV-infected marmoset cell line B95-8 (American Type Culture Collection-ATCC CRL 1612) was used as a source of EBV. Cells were grown until a concentration of 10^6 cells/ml, and the EBV-containing supernatant was harvested by centrifugation at 250 g to remove cells and debris. The supernatant was then passed twice through a 0.45 µM membrane filter (Millex, Millipore). This virus preparation can be kept at 4°C for several months (Neitzel, 1986).

Immediately before use, the EBV-containing supernatant was diluted 1:1 with fresh medium containing 50 µg/ml gentamicin, 10% fetal calf serum and 2 mM L-glutamine. Total PBMCs were resuspended in this solution at a concentration of $2x10^6$ cells/ml. Two ml-cultures were established. Half of the culture medium was replaced at the latest 24 hours after starting of the culture by addition of fresh RPMI 1640 with 20% fetal calf serum, 2 mM L-glutamine, 50 µg/ml gentamicin (final concentration) and 2 µg/ml (final concentration) of cyclosporin A (Sandimmun, Sandoz). The medium was then refreshed once a week by removing half of the supernatant and replacing it by fresh medium containing 1 µg/ml of cyclosporin A for 4 weeks (Neitzel, 1986).

DNA samples from each available member of the families were extracted according to the salting out procedure either from fresh blood samples or from continuous B lymphocyte cell lines immortalized with EBV, as described above. In the case of deceased patients, where possible, DNA was extracted from a paraffin-embedded myocardial tissue sample (Jackson *et al.*, 1991).

Microsatellite amplification

Microsatellite amplification by PCR analysis was carried out in 50 µl of a solution containing 10 mmol/l Tris pH 8.0, 50 mml/l KCl, 1.5 mmol/l $MgCl_2$, 0.01% gelatin, 200 mmol/l each dNTP, 0.1 mmol/l both primers, 200 ng of template DNA and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Roche Molecular Systems; Branchburg, NJ). Denaturation of the template at 94°C for 5 min was followed by 40 cycles in an Eppendorf mastercycler 5330, according to the following conditions: 45 sec at 94°C, 30 sec at the annealing temperature and 30 sec at 72°C, followed by 5 min of final extension at 72°C.

Annealing temperatures varied from 50°C to 68°C, according to the different primers pairs. The PCR products were subsequently resolved by a long run on a 10-12% polyacrylamide native gel and visualised by ethidium bromide staining.

Oligonucleotides were synthesized by the ICGEB Oligonucleotide Synthesis Service on an Applied Biosystem 380B synthesizer, or purchased from Primm srl (Milano, Italy) and Gibco BRL Life Technologies LTD (Paisley, Scotland).

Linkage analysis

All polymorphisms were scored without knowledge of phenotypic data and by at least two independent observers. SIMLINK computer program version 4.1 (Plougham and Boehnke, 1989) was used to test the appropriate linkage power of family FDC1. Two point and multipoint linkage analyses were performed using the PC version of the MLINK, LODSCORE and LINKMAP options of the LINKAGE package (Version 5.2) (Lathrop *et al.*, 1984; Lathrop and Lalouel, 1985). Where present, loops derived by consanguineous marriages were broken by MAKEPED option of the same package. The HOMOG program (ver. 3.3) (Ott, 1991) was used to test genetic homogeneity.

Autosomal Dominant Familial Dilated Cardiomyopathy

DNA samples from each available member of the families were extracted according to the salting out procedure either from fresh blood samples or from continuous B lymphocyte cell lines immortalized with EBV, as described above.

Polymorphic microsatellite sequences (CA repeats) with more than 70% of heterozygosity were used. Oligonucleotide sequences derived mainly from Généthon maps (Dib *et al.*, 1996; Gyapay *et al.*, 1994), or Group NCM (NIH/CEPH Collaborative Mapping Group 1992), except for loci D9S199 (Graw and Kwiatkowski, 1993), D9S104 (Wilkie *et al.*, 1992) and D9S66 (Kwiatkowski *et al.*, 1992). Other sequences were obtained from the Genbank internet web site and belonged to different sources.

Linkage analysis

The linkage analysis was performed as descibed above. Two loops derived by consanguineous marriages in family FDC1 were broken by MAKEPED option of the same package. The genetic homogeneity was tested for FDC1. Family members under 16 years were excluded from the linkage study, to avoid the low penetrance in this age group. According to the family data, the penetrance was assumed to be 0.95. A FDC gene frequency 0.0001 was derived from population surveys (Codd *et al.*, 1989; Michels *et al.*, 1992). Microsatellite allele frequencies were calculated from the families. No sex difference in the recombination events was assumed. All computations were performed under two models, according to the phenotypic assignment of individuals IV-24 and V-24 of FDC1 and individual III-1 of FDC3 as unknown or as affected, respectively.

In order to test the stability of the obtained lod values, the maximum two point lod scores were recalculated applying the sensitivity test (Hodge and Greenberg, 1992), varying the penetrance from 100% to 75% or setting marker allele frequencies to equal. The support interval (confidence limits = 90%) was calculated using the "1-unit-down" method (Ott, 1991). Due to the computer/software constraints, the number of alleles at each locus was reduced for multipoint analyses, without loss of information with respect to the FDC gene.

Characterization of candidate genes for the FDC locus

A total of eight genes mapping within the region identified by linkage analysis spanning the D9S153-D9S152 interval, or very close to that, were studied as possible candidate genes for FDC.

The nucleotide sequence of the tropomodulin cDNA was directly determined (see below), while linkage of the disease locus to the the b-tropomyosin (TPM2), phosphoglucomutase-5 (PGM5), protein kinase, cAMP-dependent, catalytic, gamma (PRKACG), Friedreich Ataxia (FRDA). collage type XV, alpha-1 polypeptide (COL15A1), annexin I (ANX1), and beta-tropomyosin (TPM2) genes was assessed by the study of inheritance of highly polymorphic microsatellites mapping intragenically or in close proximity of the genes. The beta-tropomyosin (TPM2) gene locus was tested with the Genethon marker D9S1791 which maps to that region. The phosphoglucomutase-5 (PGM5) gene mapped to the centromeric region of chromosome 9 (Edwards *et al.*, 1995) was studied with the markers Genethon D9S1791, D9S50 and D9S15 to cover the whole centromeric region of chromosome 9. The protein kinase, cAMP-dependent, catalytic, gamma (PRKACG) locus was covered with D9S202 and D9S15. The Friedreich ataxia (FRDA) locus was checked with marker D9S202 (Pandolfo *et al.*, 1993) which is very closed to the gene also according to the integrated maps released by the

NCBI (National Centre for Biological Information-NIH, Bethesda, MD). Collagen type XV, alpha-1 polypeptide (COL15A1) and Annexin I (ANX1) gene loci were tested wtih the Genethon markers D9S15, D9S166, D9S301, D9S175, D9S967 and D9S768.

Determination of the cDNA sequence of tromomodulin was carried out from total RNA extracted from B-lymphoblastoid cell lines from patients IV-1, IV-14 and IV-29 of family FDC-1 according to a published procedure (Chomczynski and Sacchi, 1987). The cDNA was obtained by priming with an oligo-dT primer followed by reverse transcription, accordin to the following conditions: 10 min annealing at 65°C, 1 hr extension at 37°C, followed by 5 min at 95°C. Conditions for PCR amplification were: 30 sec at 94°C, 30 sec at 56°C, 1 min at 72°C, followed by 5 min of final extension at 72°C.

PCR was performed using the following primers from the 5' to the 3' ends of the complete cDNA sequence of the tropomodulin gene:

tmod-1 (5'-TCAGGAGACACAGACAAGTT-3');

tmod-2 (5'-CGAATTTGAGAAGTGTTGCG-3');

tmod-3 (5'-GGCAACAAAGTGGAAATGGA-3');

tmod-4 (5'-CATTTGCCCACTGTGCAATA-3');

tmod-5 (5'-GAATGCCAACCTTATGATGG-3'); and

tmod-6 (5'-ATTGCAGTTTTCAGCGATGC-3').

The oligonucleotide sequences were designed with the Amplify^{RM} computer program, in order to match to the sequence of the TMOD cDNA (Sung *et al.*, 1992) present in the GenBank database and to have all the same theoretical temperature of melting (acting as annealing temperature).

Three overlapping fragments were obtained for each patient. Primer pair tmod-1/2 gave the expected PCR fragments of 976 bp, pair tmod-3/4 gave the expected PCR fragment of 1049 bp, pair tmod-5/6, gave the expected PCR fragment of 765 bp.

Polymerase chain reaction was performed in an Eppendorf mastercycler 5330, according to the following conditions: 5 min denaturation at 95°C, 45 sec annealing at 58°C, 90 sec extension at 72°C, followed by 5 min°of final extension at 72°C. The amplified fragments were eluted from polyacrylamide gels and directly cloned in the pCR^{TM}II vector (Invitrogen, San Diego, CA).

Sequence analyses were performed on plasmid DNA extracted from individual bacterial clones by the dideoxynucleotide chain termination method using a DNA sequencing kit (Pharmacia, Uppsala, Sweden) based on the utilization of T7 DNA polymerase and (α^{35}S)-dATP; labelled ^{35}S-deoxynucleotides were obtained from Amersham International plc (Amersham, UK). Sequence data were obtained by extension of the universal primer of the vector and by using the specific internal primers listed in table II.

To avoid misinterpretations due to mutations introduced by *Taq* polymerase misincorporation during the PCR amplification process, each sequencing run was repeated and verified by sequencing a newly amplified fragment obtained from new RT-PCR reactions.

X-Linked Dilated Cardiomyopathy

DNA samples from each available member of the families were extracted according to the salting out procedure either from fresh blood samples or from continuous B lymphocyte cell lines immortalized with EBV, as described above. In the case of the deceased patient II-1 belonging to the XLDC-1 family, DNA was extracted from a paraffin-embedded myocardial tissue sample (Jackson *et al.*, 1991).

Multiplex PCR as DMD mutations diagnostic tool for XLDC

The screening for deletions of the dystrophin gene was performed using multiplex PCR, according to methods described by (Chamberlain *et al.*, 1988 and Beggs *et al.*, 1990). Amplifications of the first muscle exon, muscle promoter, brain promoter and second exon were carried out as reported (Boyce *et al.*, 1991; Klamut *et al.*, 1990; Koenig *et al.*, 1987; Muntoni and Strong, 1989).

Haplotype analysis for XLDC

For the haplotype analysis, the polymorphic microsatellite sequences (CA repeats) DYS-I, DYS-II, DYS-III, DYS MSB, DYS MSA, DXS 997, DX992 were PCR amplified using described conditions (Frener *et al.*, 1991; Saad *et al*, 1993). PCR products were resolved by a long run on a 10-12% polyacrylamide native gel and visualized by ethidium bromide staining.

Reverse transcription and PCR of the muscle promoter, first exon/intron and C-ter region of the dystrophin gene

Total RNA was isolated from frozen samples according to a published procedure (Chomczynski ans Sacchi, 1987) and cDNA synthesis was performed using random hexanucleotide primers. PCR was carried out in a reaction volume of 25 µl containing the cDNA template (2 µg) and oligonucleotide primers designed to amplify the muscle isoform of dystrophin (forward oligonucleotide in the first muscle exon and reverse oligonucleotide located in exons 2 or 4) and the Dp71 transcript (Muntoni *et al.*, 1995a; Muntoni *et al.*, 1995b). PCR reactions (25 µl) utilized 0.5 units *Taq* DNA polymerase, 0.25 mM each primer, 200 mM each dNTP, in 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$. After 10 min denaturation at 94°C, PCR amplification was carried out with the following cycle profile: denaturation at 94°C for 30 sec; annealing at 58°C for 60 sec; extension at 72°C

for 120 sec; 26 cycles. Nine ml of the reaction were resolved on 2.5% agarose gels containing 0.2 g/ml of ethidium bromide.

PCR-SSCP analysis

For point mutations screening, the region including the muscle promoter, the first muscle exon and 250 bp of the first intron of dystrophin were amplified by PCR using serial pairs of primers to obtain products of about 100 to 300 bp. PCR amplifications were carried out in 50 µl of a solution containing Tris-HCl 10 mM (pH 8.0), KCl 50 mM, $MgCl_2$ 1.5 mM, gelatin 0.01%, each dNTP 200 mM, each primers 0.1 µM, 200 ng of template DNA and 2.5 units of *Taq* DNA polymerase (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ), with 40 repetitions of the following cycle: 30 sec at 94°C, 30 sec at the annealing temperature and 30 sec at 72°C. Annealing temperatures varied from 56°C to 62°C, according to the different primer pairs. The PCR product encompassing the 3' end of the muscle first exon and the beginning of intron 1 was obtained using the following primers: forward primer, 5'-TATCGCTGCCTTGATATACA-3'; reverse primer, 5'-ACTAAACGTTATGCCACAGT-3', with an annealing temperature of 60°C.

PCR-SSCP samples were prepared by denaturing 5 µl of PCR product with 7 µl of formamide dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) at 94°C for 8 min. After cooling in ice, the samples were quickly loaded on 10% -20% non-denaturing polyacrylamide gels with or without glycerol and run for 12-16 hours at room temperature or at 4°C. DNA fragments on SSCP gels were visualized by silver staining using a commercial procedure (BioRad, Richmond, VI).

Cloning , sequencing , and restriction analysis of the PCR products from the dystrophin gene 5' end

The PCR amplified fragments obtained as described above were eluted from polyacrylamide gels and directly cloned in a commercial vector (TA

Cloning Kit, Invitrogen). Sequence analysis was performed on plasmid DNA extracted from at least three individual bacterial clones by the dideoxinucleotide chain termination method using a T7-based DNA sequencing kit (Pharmacia). Sequence data were obtained for both strands of the insert by extension of the universal and reverse primers of the vector, as well as by using internal primers.

The mutation found at the 5' splice site of the first dystrophin intron predicts the generation of a new restriction site for *Mse* I. The restriction enzyme was purchased from New England Biolabs (Beverly, MA). The 110 bp fragment encompassing the mutation was digested with this enzyme to produce two fragments of 60 and 50 bp, that were visualized by electrophoresis on an ethidium bromide-stained 8% polyacrylamide gel.

Dilated cardiomyopathy with skeletal muscle involvement (MDDC)

Clinical and genetic features

In our family study, 3 families presented DC associated with subclinical skeletal muscle involvement resembling XLDC, but with *autosomal dominant* pattern of transmission. The clinical characteristics of the disease did not differ from that found in other forms of DC.

The family pedigree were constructed, and two to three generations were studied **(Fig. 6)**. Of the 22 studied subjects, 8 were affected, 10 were unaffected, and the remaining, who did not fulfil the criteria for the diagnosis of DC but presented minor clinical, ECG or echocardiographic abnormalities, were defined as "unknown". The main features of the three families are summarised in **Table 4.**

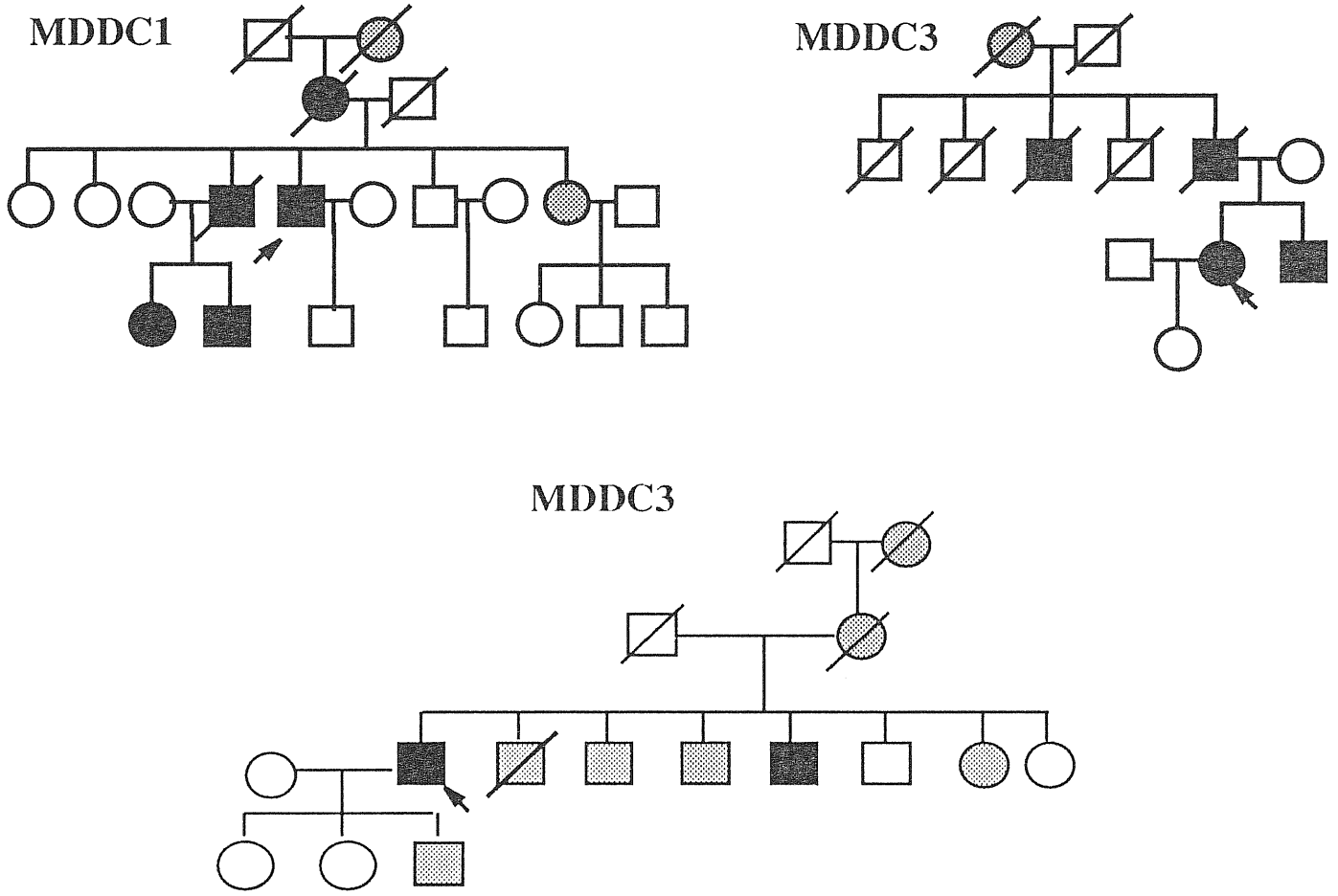


Figure 6. Pedigrees of three families studied for Autosomal Dominant Familial Dilated Cardiomyopathy with subclinical myopathy (MDDC). Affected individuals are indicated by filled circles (females) or filled squares (males), whereas unaffected are indicated by open circles and squares. Individuals with equivocal or suspected DC are shown in gray.

PATIENT POPULATION

- 42 families with FDC prospectively studied
- 5 families (12%) with signs of skeletal muscle involvement in the affected family members
- 34 family members analyzed:

affected	13
healthy	15
unclassifiable	6

- 2 patterns of transmission identify at least 2 different forms:

X-linked	⇒	XLDC
autosomal dominant	⇒	MDDC*

*(muscle disease with dilated cardiomyopathy)

MDDC main features:

- familial form of dilated cardiomyopathy with autosomal mode of inheritance
- both females and males affected, with variable age at onset
- absence of overt signs of skeletal myopathy
- possible mild increase of muscle CK in affected relatives
- the disease gene (or genes), is still unknown.

Table 4. Summary of the population study of FDC with myopathy and main features of the newly identified MDDC form

Hystology and immunocytochemistry

In order to investigate and characterize the skeletal muscle morphology changes in MDDC patients, muscle biopsy of the quadriceps from each patient were obtained, after informed consent. Frozen sections were prepared and stained either according to standard histological procedures or with immunohistochemical examinations, performed using a biotin-streptavidin Texas-red method.

The immunostaining was done using a panel of antibodies to all the muscle cell compartments (extracellular matrix, sarcolemma, sub-sarcolemma and cytoskeleton) using antibodies against dystrophin (N-ter, C-ter and mid rod domain), desmin, β-dystroglycan (43 DAG), α-sarcoglycan (50 DAG), β-1-laminin and fetal myosin, as described (Muntoni *et al.*, 1993).

Candidate linkage analysis for MDDC

Thirteen members of the MDDC-1 family were investigated by linkage analysis. The remainding two families were excluded because not enough informative.

Linkage analysis was performed using highly polymorphic markers derived mainly from Généthon maps. They were chosen for their location either intragenic or very close to the gene of interest. A total of 32 dinucleotide polymorphisms (microsatellites) were analysed. These markers were linked or very closed to the following genes coding for cytoskeleton and extracellular matrix proteins: actinin alpha 2 (1q42-q43), actin gamma 2 (2p13.1), actin alpha cardiac (15q14), titin (2q31-q32), nebulin (2q31-q32), dystroglycan alpha (3p21), gamma (13q12), myosin light chain polypeptide (3p21), myosin cardiac beta polypeptide 7 (14q12), sarcoglycan, alpha (adhalin; 17q12-q21.33), beta (4q12), gamma (13q12) and delta (5q33), catenin alpha 1 (5q31), laminin alpha 2 (merosin; 6q22-q23), laminin beta 1 (7q31.1-q31.3), utrophin (6q24), filamin 2 (7q32-q35), syntrophin beta 1 (8q23-q24), syntrophin

beta 2 (16q22-q23), vinculin (10q22.1-q23), calpain (15q15.1-q21.1), troponin I (19p13.2-q13.2) and collagen VI (21q22.3). Moreover, with polymorphic markers we analysed the loci for: CDDC (1p11-q11), LGMD1B (1q11-q21), LGMD2B (2p13.3-p13.1), CDDC (3p22-p25). FSHD1A (4q35), LGMD1A (5q22.3-q31.3), Friedrich ataxia locus (9q13-q21.1) and the locus for the myotonic dystrophy (19q13.3).

RESULTS-PART I

MOLECULAR GENETICS OF FDC

To understand the molecular basis of the genetic form of dilated cardiomyopathy, molecular genetic studies were undertaken. Taking into account the phenotypic heterogeneity and the expected genetic heterogeneity, the research was addressed toward the identification of the disease gene in single kindreds.

AUTOSOMAL DOMINANT FORM OF DILATED CARDIOMYOPATHY

Genetic heterogeneity and penetrance in FDC

The autosomal dominant trait represent the most frequent form of FDC. However, the existence of genetic heterogeneity could not be excluded within this subgroup. For this reason, a more conservative molecular genetic approach requires the analysis of a single large family.

Single large kindred sufficiently informative for linkage analysis are rare, because of the absence of early markers of disease, of the high mortality, and of the reduced and age-related penetrance. The problem of penetrance could lead to misclassifications of the clinical status in actual carriers, especially in young family members within the third decade of life, clearly reducing the number of family members suitable for the study. Moreover, the real values of penetrance are not known, since is not possible by now to confirm at the molecular level the carrier status. The evaluation of penetrance is initially performed on the basis of clinical observations (Ott, 1991). The age-related penetrance for AD-FDC was calculated in our study

families: < 20 years: 10%, 20-30: 34%,30-40: 60%, >40: 90%, whereas an overall penetrance assumed to be around 80% was reported by other authors (Pastores *et al.*, 1991).

Phenotypic assessment of AD-FDC.

Eighty members of a single family with AD-FDC, initially identified in 1987, were investigated (**Fig. 7**).

The selection criteria were the presence of dilated cardiomyopathy with autosomal dominant pattern of transmission, the absence of any clinical or histological sign of skeletal muscle involvement, and the sufficient informativeness for a linkage study. Full invasive evaluation was performed in 9 patients. In the remaining patients, coronary artery disease was excluded by clinical criteria and non-invasive testing. In the proband of family FDC1 (IV-29), normal skeletal muscle biopsy and karyotype analysis demonstrated the absence of subclinical skeletal muscle involvement and of chromosomal aberrations, respectively.

From 1991 to 1994, all affected and unaffected members of the studied families were periodically examined, to minimise the risk of misdiagnosis. The affection status was established without knowledge of genotype, and at the end of the last follow up (August 1997) the criteria for the phenotypic assessment were reviewed in all family members.

After the last follow up, 14 members of family FDC1 were considered to be affected. Family members with subclinical cardiac impairment, such as isolated frequent and repetitive ventricular arrhythmias, or left ventricular dilatation and dysfunction in the presence of arterial hypertension and ventricular hypertrophy, were considered of unknown status for linkage analysis. Finally, patients with other defined cardiac affections, in particular with clinical and laboratory evidence of ischemic heart disease, were considered as unaffected.

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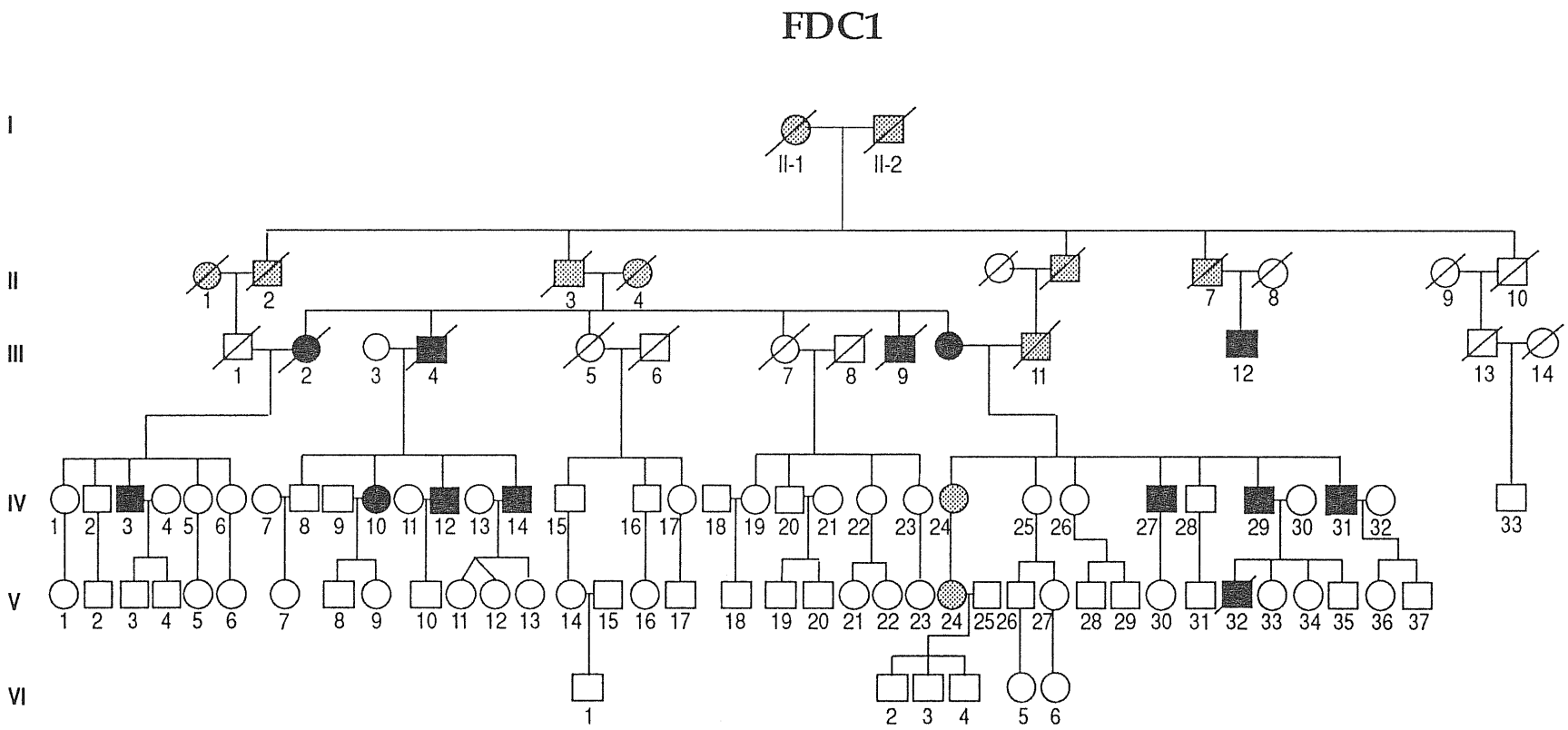

Figure 7. Pedigree of family FDC1, consistent with autosomal dominant inheritance. Affected individuals are indicated by filled circles (females) or filled sqyares (males), whereas unaffected are indicated by open circles and squares. Individuals with equivocal or suspected DC are shown in gray.

Two other kindreds with autosomal dominant pattern of transmission (FDC2 and FDC3) were subsequently identified out of a group of 19 families with dilated cardiomyopathy; the remaining 17 families were excluded from linkage study because of not sufficient informativeness, different pattern of transmission or different clinical characteristics. Diagnosis of dilated cardiomyopathy and clinical evaluation were made according to the same criteria used for family FDC1, identifying 8 affected and 3 unaffected relatives in families FDC2 and FDC3. One case (III-1) of family FDC3, with isolated left ventricular dilatation, was considered as unknown. The groups of affected and unaffected members enrolled in the study did not differ significantly in age and sex distribution.

Whole-genome random screening and first construction of an exclusion map

The linkage study was initiated in kindred FDC1, which had been proved to have a sufficient linkage power (Krajinovic *et al.*, 1994). Random screening was performed with 251 highly polymorphic microsatellite sequences dispersed throughout the genome. For all tested markers, two-point lod scores were calculated and exclusion maps were constructed. The distance between markers was based on Généthon microsatellite maps (Gyapay *et al.*, 1994) CHLC, Cooperative Human Linkage Consortium, map (CHLC, 1994). The sum of the unexplored regions located between two adjacent excluded intervals, and not larger than 5-10 cM, was around 150 cM. Thus, before establishing linkage, about 95% of the genome of 21 autosomes was excluded (Gyapay *et al.*, 1994).

Initial identification of a FDC region linked to chromosome 9

The first result suggestive of linkage to chromosome 9 was obtained analysing a dinucleotide repeat at the D9S156 locus (Z_{max} 2.46 at $\theta = 0$) (Gyapay *et al.*, 1994). Seventeen additional markers spanning the entire chromosome 9

were then tested, and, finally, the two smaller families with the same clinical features (FDC2 and FDC3) were included in the study to provide additional evidence. Two point linkage analysis revealed the maximal probability region within two loci on chromosome 9q, D9S153 and D9S152 (Gyapay *et al.*, 1994). Cumulative and by family pairwise lod scores for 15 markers on chromosome 9 are presented in **Table 5**. The maximum cumulative two point lod scores were obtained for loci D9S153 ($Z_{max} = 3.69$, $\theta = 0.08$) and D9S152 ($Z_{max} = 3.21$, $\theta = 0.09$).

Considering individuals FDC1 IV-24, V-24 and FDC3 III-1 with subclinical cardiac impairment as affected, linkage was confirmed for the two loci D9S153 (Z_{max} 3.18, $\theta = 0.011$) and D9S152 (Z_{max} 3.46, $\theta = 0.08$). In particular, a significant lod score of 3.18 ($\theta = 0.09$) was obtained with the single large kindred FDC1 in D9S152.

Lod score values remained significantly positive (> or = to 3) also after modifying the penetrance from 100% to 75%, as well as after changing allele marker frequencies to equal. Changes in the affection status, when performing the sensitivity test (Hodge and Greenberg 1992), did not affect the lod values. The null hypothesis of locus homogeneity (Ott, 1991) was confirmed ($p = 0.000041$).

The reconstruction of the haplotypes for 9 markers of chromosome 9 was performed by minimising the recombination events between the markers **(Fig. 8)**. Obligate recombination events in three affected individuals from family FDC1 (III-4, IV-29, IV-31) and one from family FDC2 (IV-2) were identified at the loci D9S153 and D9S257, thus setting the boundaries for the disease locus. Telomeric and centromeric cross-overs that occurred in the other individuals are outside of this region **(Fig. 9)**.

The genotype of unaffected individual IV-8 (42 years old) is concordant with disease status for both D9S153 and D9S152 loci. This divergence could derive from phenotypic misclassification, recombination events between the

Table 5. Cumulative and by family lod scores for fifteen markers of chromosome 9

MARKER	KINDRED	Z at θ =							Z_{max}
		0.00	0.01	0.05	0.10	0.20	0.30	0.40	
D9S156	FDC1	2.46	2.42	2.27	2.06	1.63	1.15	0.61	
	FDC2	-0.53	-0.47	-0.30	-0.17	-0.06	-0.01	0.00	
	FDC3	0.20	0.20	0.17	0.13	0.08	0.03	0.01	
	Cumulative	2.13	2.15	2.14	2.02	1.65	1.17	0.62	2.16
D9S157	FDC1	-1.40	1.20	2.03	2.29	2.16	1.63	0.84	
	FDC2	-2.30	-0.53	0.08	0.28	0.36	0.39	0.16	
	FDC3	0.19	0.18	0.16	0.14	0.09	0.04	0.01	
	Cumulative	-3.51	0.85	2.27	2.71	2.61	2.06	1.01	2.76
D9S162	FDC1	-17.76	-4.13	-1.29	-0.15	0.63	0.71	0.47	
	FDC2	0.53	0.53	0.50	0.46	0.38	0.27	0.15	
	FDC3	0.16	0.15	0.13	0.11	0.07	0.03	0.01	
	Cumulative	-17.07	-3.45	-0.66	0.42	1.08	1.01	0.63	0.11
D9S171	FDC1	-15.45	-4.39	-1.52	-0.35	0.50	0.65	0.45	
	FDC2	-0.86	-0.81	-0.62	-0.45	-0.22	-0.09	-0.02	
	FDC3	0.20	0.19	0.17	0.14	0.08	0.04	0.01	
	Cumulative	-16.11	-5.01	-1.97	-0.66	0.36	0.60	0.44	0.6
D9S104	FDC1	-6.31	-1.91	-0.79	-0.34	-0.01	0.07	0.06	
	FDC2	0.41	0.41	0.40	0.39	0.34	0.26	0.15	
	FDC3	0.24	0.23	0.19	0.14	0.06	0.02	0.00	
	Cumulative	-5.66	-1.27	-0.20	0.19	0.39	0.35	0.21	0.39
D9S175	FDC1	-7.98	-1.34	0.60	1.27	1.53	1.26	0.70	
	FDC2	0.74	0.72	0.67	0.60	0.46	0.31	0.16	
	FDC3	0.16	0.15	0.11	0.08	0.02	0.00	0.00	
	Cumulative	-7.08	-0.47	1.38	1.95	2.01	1.57	0.86	2.08
D9S153	FDC1	-5.37	1.49	2.57	2.76	2.47	1.80	0.92	
	FDC2	1.05	1.02	0.93	0.82	0.60	0.38	0.18	
	FDC3	0.13	0.12	0.11	0.08	0.05	0.02	0.01	
	Cumulative	-4.19	2.63	**3.61**	**3.66**	**3.12**	2.20	1.11	**3.69**
	90% confidence limits: 0.011-0.25								
D9S152	FDC1	-0.74	2.11	2.79	2.92	2.61	1.93	0.99	
	FDC2	0.27	0.26	0.23	0.19	0.12	0.06	0.01	
	FDC3	0.13	0.12	0.11	0.09	0.05	0.02	0.01	
	Cumulative	-0.34	2.49	**3.13**	**3.20**	2.78	2.01	1.01	**3.21**
	90% confidence limits: 0.006-0.27								
D9S257	FDC1	-18.40	-6.13	-2.95	-1.49	-0.21	0.25	0.28	
	FDC2	-4.81	-1.00	-0.38	-0.16	-0.04	-0.01	-0.01	
	FDC3	-0.09	-0.09	-0.07	-0.06	-0.03	-0.01	0.00	

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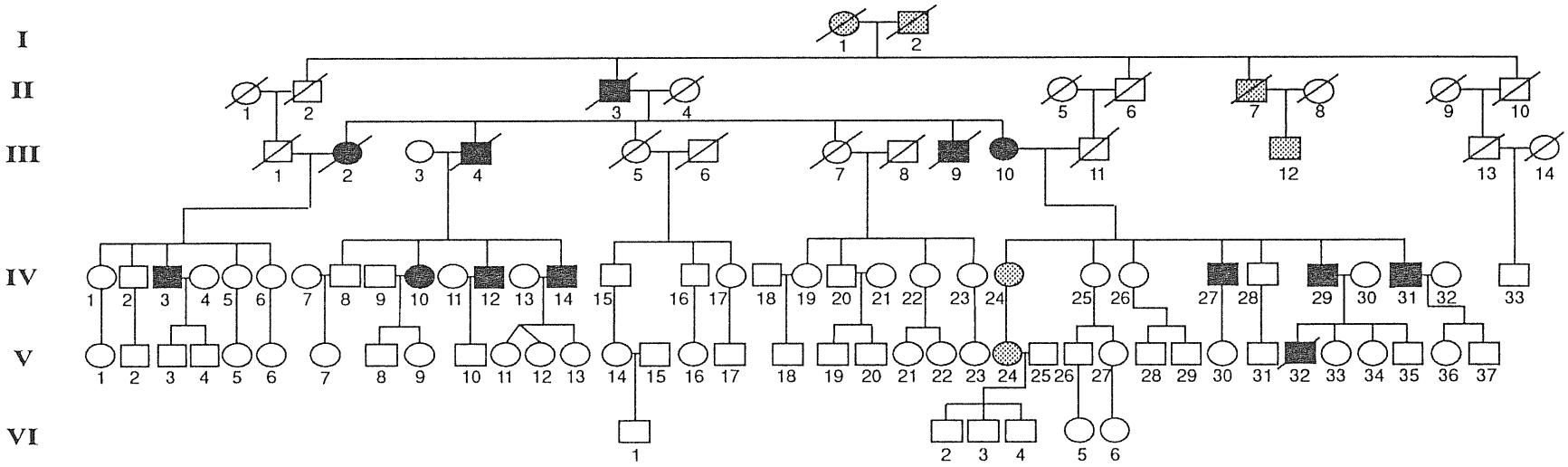

B

LOCUS	IV-1	IV-3	IV-5	IV-6	III-1	III-2	III-3	III-4	IV-8	IV-10	IV-12	IV-14	II-4	II-3	III-5	III-7	III-9	III-10	IV-24	V-24	IV-25	IV-26	IV-27	IV-28	IV-29	V-33	IV-31	III-11
D9S199	11	41	11	11	41	11	11	11	11	11	11	11	11	11	11	11	11	11	12	22	11	12	21	11	12	12	12	12
D9S168	11	12	11	21	11	12	45	12	25	24	24	41	11	21	11	12	12	12	13	17	13	16	62	23	26	56	26	36
D9S285	33	32	33	33	33	32	25	42	25	22	22	24	34	23	33	33	32	32	33	33	33	31	12	23	21	11	21	31
D9S156	21	13	21	21	11	23	32	33	32	33	33	33	23	33	23	23	23	23	22	24	22	23	33	32	33	13	33	23
D9S157	21	11	21	21	11	21	13	41	13	11	11	14	24	12	22	22	21	21	21	24	21	21	61	11	16	56	16	16
D9S162	21	13	21	21	11	23	21	43	31	31	32	24	24	32	23	22	22	23	25	22	25	25	53	35	25	55	35	55
D9S171	21	15	21	21	11	25	75	35	55	55	57	53	23	52	35	22	22	35	32	32	32	32	25	52	34	24	54	24
D9S104	13	11	13	13	13	11	22	11	12	12	12	21	11	12	11	12	12	11	14	12	14	14	41	14	13	33	13	43
D9S175	11	62	11	11	61	12	43	12	23	24	23	31	11	23	12	13	12	12	17	11	17	17	72	17	15	85	25	75
D9S153	12	32	12	12	32	12	12	12	21	22	22	11	11	21	11	11	12	12	11	15	11	11	12	11	14	24	24	14
D9S152	11	13	11	11	11	13	53	13	35	33	33	51	21	31	11	21	23	13	33	35	13	13	33	13	34	34	34	34
D9S257	34	41	34	14	43	31	56	45	56	56	56	54	43	15	35	41	41	31	11	18	11	31	11	31	12	52	32	12
D9S283	14	51	15	15	45	11	41	41	14	14	14	44	41	11	11	41	41	11	13	11	13	13	31	13	12	12	12	32
D9S176	24	12	21	21	41	22	33	44	43	43	43	34	42	24	42	42	42	22	23	33	23	23	32	23	22	32	22	32
D9S277	65	35	53	55	53	65	77	65	57	57	57	76	63	25	62	62	65	32	24	49	24	34	42	34	21	11	31	41

Figure 8. Pedigree of family FIDC1, consistent with autosomal dominant inheritance. Individuals are indicated by generation and pedigree number. The affection status is indicated by solid symbols (affected), clear symbols (unaffected) and stippled symbols (unknown). (B) Haplotypes of the key members of FIDC1, resulting from the analysis of 15 informative microsatellite markers located on chromosome 9: the grey boxed areas represent the haplotype cosegregating with the disease. The haplotypes of individuals II-3 and II-4 are inferred. For the discrepancy of family member IV-14 see text.

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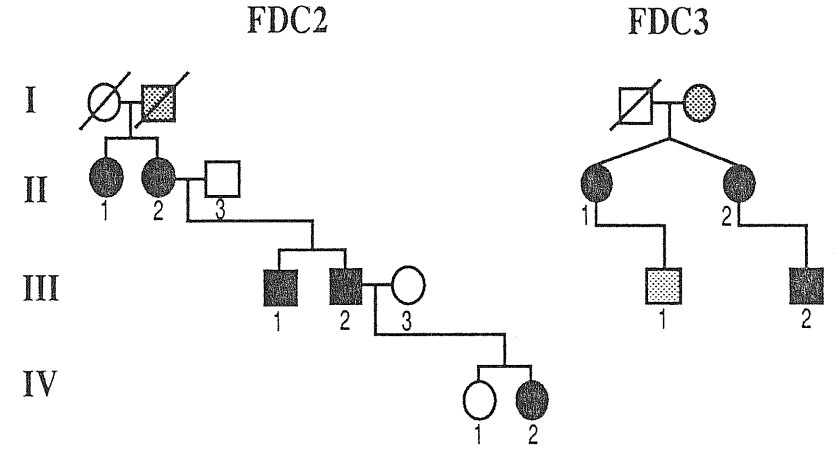


B

LOCUS	II-1	II-2	III-1	III-2	III-3	IV-1	IV-2	II-1	IV-1	II-2	III-2
D9S199	23	21	13	23	23	33	32	11	12	11	12
D9S168	25	11	13	12	13	11	31	32	21	32	22
D9S285	11	41	45	45	16	14	64	11	11	11	11
D9S156	22	22	21	21	23	22	32	31	11	31	11
D9S157	45	25	23	23	11	13	12	62	21	62	21
D9S162	35	33	35	35	55	55	53	45	55	46	55
D9S171	25	24	24	22	25	22	22	23	32	23	22
D9S104	14	11	14	14	32	34	31	15	51	25	53
D9S175	44	44	45	48	74	78	44	11	11	21	13
D9S153	23	23	21	21	42	41	22	12	23	12	23
D9S152	66	66	66	66	36	36	66	31	12	31	23
D9S257	16	15	12	12	43	32	32	21	12	11	13
D9S283	23	22	21	21	21	11	11	12	22	12	22
D9S176	23	21	23	23	43	33	33	21	23	21	14
D9S277	22	24	24	21	23	31	31	12	14	12	23

Figure 9. Extension of the analysis to other families with autosomal dominant FDC. Pedigree structure of two families with autosomal dominant FDC linked to chromosome 9. Individuals are indicated by generation and pedigree number. The affection status is indicated by solid symbols (affected), clear symbols (unaffected) and stippled symbols (unknown). (B) Haplotypes of the key members of FDC2 and 3, resulting from the analysis of 15 informative microsatellite markers located on chromosome 9: the grey boxed areas represent the haplotype cosegregating with the disease.

markers and the disease gene, or reduced penetrance. Considering the reduced and age-related penetrance of FDC, the latter hypothesis seems to be most likely one. Affected individual IV-14 of family FDC1 was recombinant for the markers within the interval D9S153-D9S257. Although the possibility of phenocopies could not be excluded, this is not likely to be the case, since this patient was studied with full invasive and non invasive examinations and showed the typical features of dilated cardiomyopathy. As segregation of the disease is suggestive of monogenic disorder, an epistatic influence of some other gene(s) appears equally unlikely, though it could not be completely ruled out. A third, most likely explanation, seems to be the occurrence of a double recombination within this interval, although this hypothesis would not be supported by the high level of interference predicted in the region 9cen-9q32 [Attwood et al. 1994].

To determine the most probable location for the disease locus, multipoint linkage analysis was performed with markers D9S175, D9S153, D9S152, D9S257 and D9S283 using LINKMAP program. A multipoint lod score value of 4.2 was obtained. This result is consistent with the two point and haplotype analyses, leading to the localization of the disease locus between D9S153 and D9S152 markers **(Fig. 10)**, under either stringent or relaxed models of phenotypic assignment.

Genetic and physical maps data of the D9S153 - D9S152 interval

The region containing the disease locus, was assumed to be 15 cM according to the map positions of the CHLC map (CHLC, 1994), although there were discrepancies with the 1994 Généthon human genetic linkage map (Gyapay *et al.*, 1994), which showed a 6 cM interval between the two markers D9S153 and D9S152. This discrepancy between the Généthon map and the CHLC map was caused by the possible errors in the allotting of each marker position in those human genetic linkage maps. In fact in 1994, all the controls

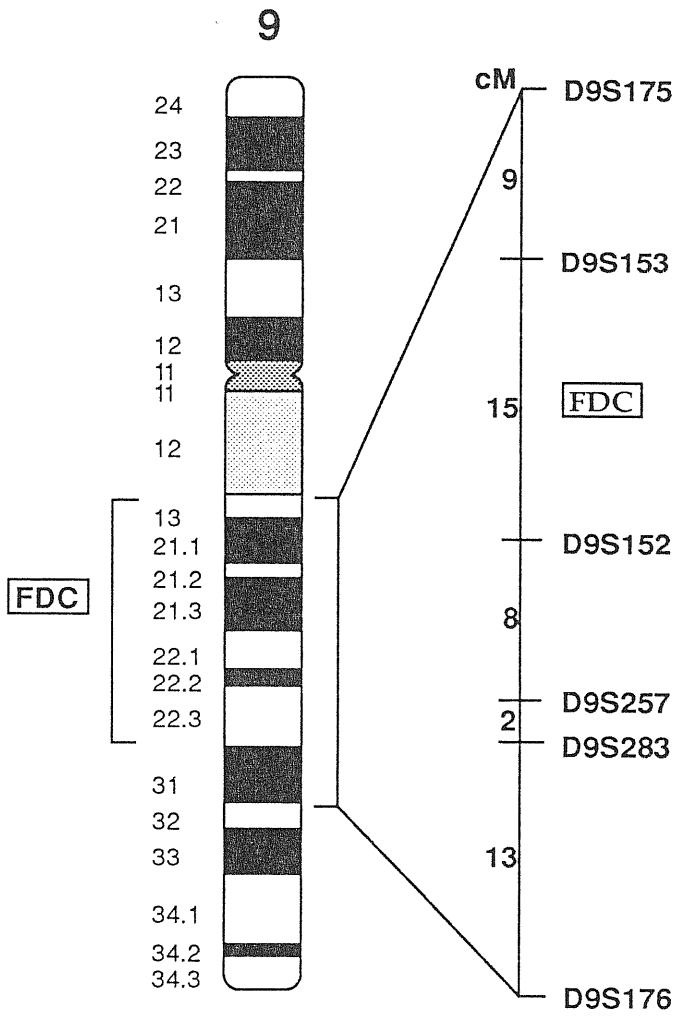


Figure 10. Ideogram of chromosome 9 showing the approximate location of the disease gene. The genetic map shows sex-average recombinant fractions between adjacent markers. The FDC locus is genetically mapped on data from Genethon [Gyapay et al., 1994] and CHLC [Murray et al., 1994].

showed some discrepancies between the obtained data and the actual relative position of some markers which have not been physically mapped (CHLC, 1994). The D9S152 marker was one of those which had not been allotted to any contig belonging to that specific region. Therefore, it was not possible to attribute a precise physical location.

Furthermore, more suitable genetic markers for narrowing the region were not available yet, and the family size was not informative enough to allow a reduction of the locus size.

Candidate genes analysis within the D9S153 - D9S152 interval

Once the linkage was established on the 9q region between the markers D9S153 and D9S152, a candidate genes approach was carried out to check if any of the genes already mapped within the region could be associated with the disease.

The candidate genes (OMIM, Online Mendelian Inheritance in Man) mapped within this region in the early 1995 were: the tropomodulin gene (TMOD), the annexin I gene (ANX1), the protein kinase, cAMP-dependent, catalytic gamma subunit gene (PRKACG) , the gene for the Friedreich Ataxia (FRDA), the collagen type XV, alpha-1 polypeptide (COL15A1), the phosphoglucomutase (PGM5) gene and the gene for the β-tropomyosin type 2 (TPM2) **(Fig. 11)**.

Among them, the most interesting gene because of the characteristics and functions of its protein product was considered to be the human tropomodulin gene.

The complete cDNA sequence of the TMOD was available in Genbank (Sung *et al.*, 1992). Total RNA was extracted from B-lymphoblastoid cell lines from the family members IV-1 (healthy), IV-14 (seek) and IV-29 (seek) according to a published procedure (Chomczynski and Sacchi, 1987). Three RT-PCR runs were performed and the products were cloned in the pCR^{TM}II

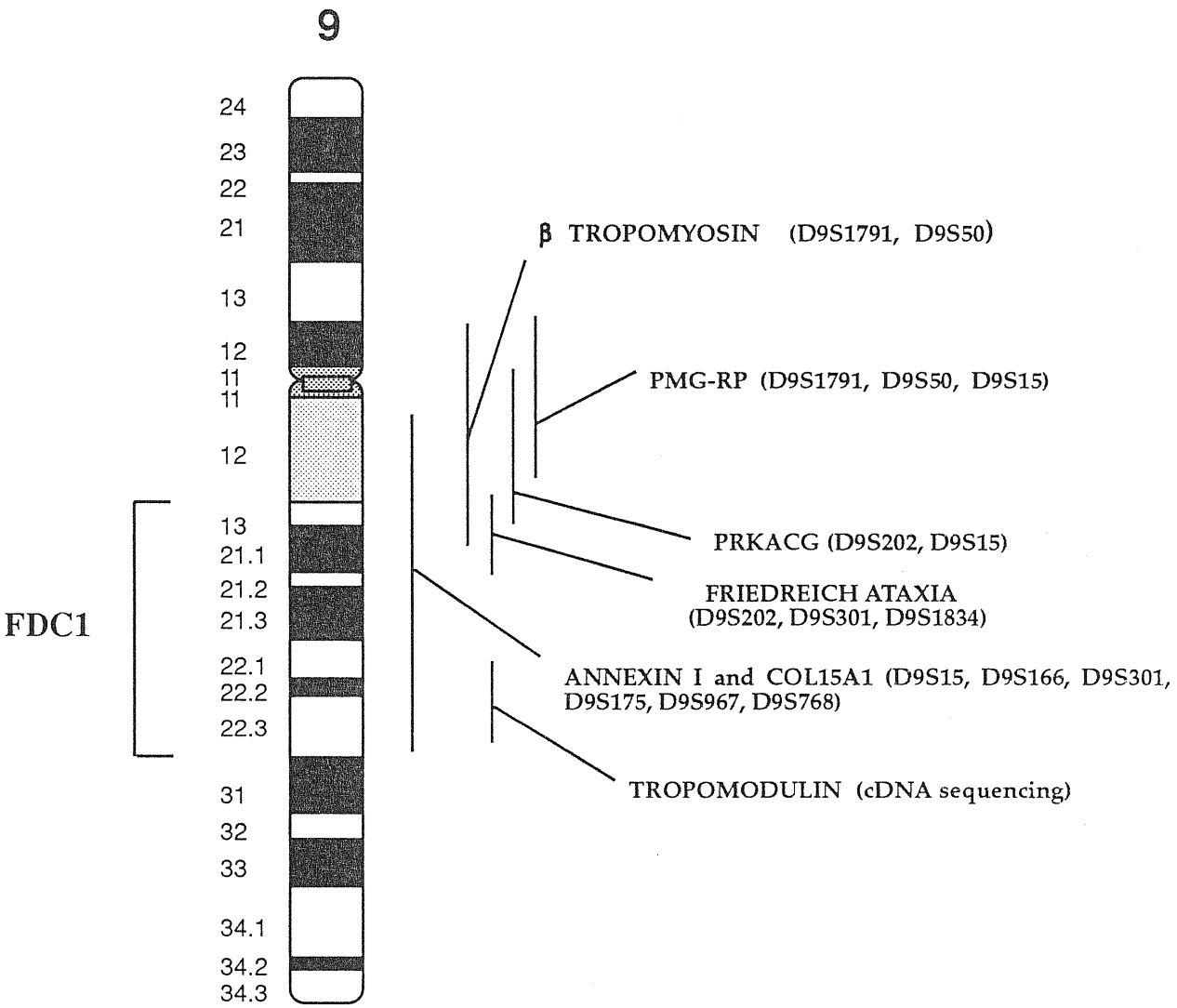


Figure 11. Candidate gene analysis for the FDC locus. All of them were excluded as disease genes.

vector of Invitrogen. Then, the entire TMOD cDNA was sequenced from several clones belonging to different growing plates. No mutations were found and the sequence was 100% matching with the published sequence (Sung *et al.*, 1992).

The other genes were tested by linkage analysis with markers present in human linkage maps but not used in the random screening of the genome or newly published intragenic or very close microsatellites which became available (as described in Materials and Methods). Also in this case, we failed in finding any of these genes linked with an odd > 1000:1.

Map refinement of the D9S153 - D9S152 interval

Since new polymorphic markers recently became available from various sources (i.e.: Withehead Institute of Technology or WIT, USA; TIGR-Consortium, USA; Sanger Human Genome Research Centre, UK), a map refinement was performed for the interval where the FDC locus was localised. Furthermore, the new 1996 Genethon map allowed the use of additional markers aligned with the genetic map (Dib *et al.*, 1996) The markers used for the refinement were flanking the D9S153 - D9S152 loci or mapping within the interval.

Flanking markers, proximal to the centromere were D9S1834, D9S301, 6 cM far apart from D9S153; D9S967 and D9S768, 2 cM far from D9S153. As internal primers, we have used D9S1780, D9S1785, D9S1867, D9S922 which are 1 cM far apart the D9S153; D9S1843 which is 2 cM distant from D9S153 and is more distal compared to the group of markers listed above. At 3 cM there are markers D9S264 and D9S167, and finally 1cM more distal there is the marker D9S152 **(Fig. 12)**.

All these markers were analysed for the two-point lod score calculation. Depending from their own informativeness, they gave the following results: markers D9S1834, D9S301 were excluding linkage at $\theta = 0$ (i.e.: for D9S301, Z=

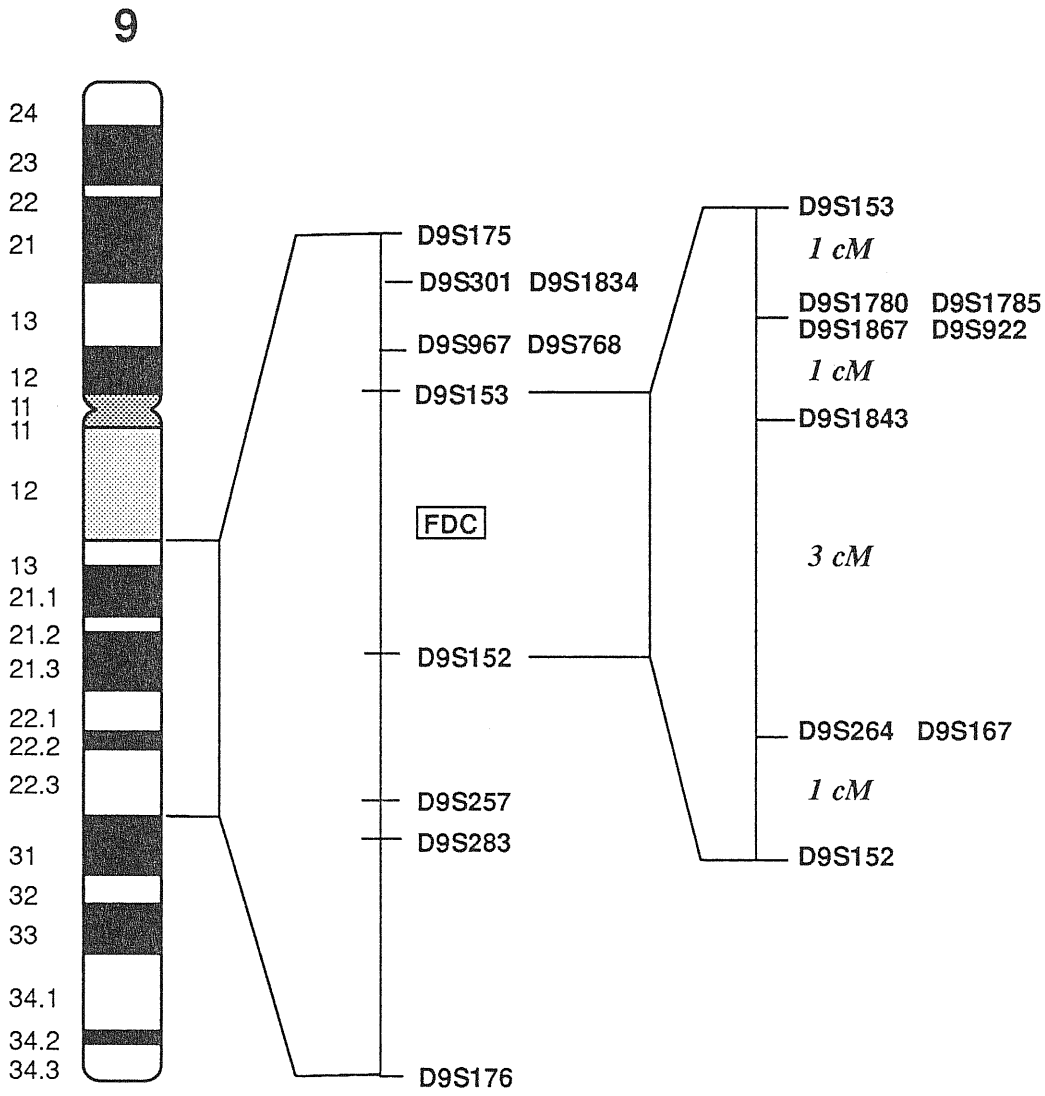


Figure 12. Refinement of the genetic map of the D9S153 to D9S152 interval of the maximum probability FDC locus, with new polymorphic markers from the latest human genetic linkage map, integrated with physical positioning data of some microsatellites [Dib et al., 1996]

-10 at θ=0), whereas they gave not informative lod score value in the interval of θ from 0.01 to 0.15 (θ = 15 has been considered limit of the calculation for this screening). D9S967 and D9S768 gave negative lod score values of Z_{max} = -2.61 and -2.38 respectively, at θ = 0.01 and D9S967 gave Z_{max} = -1.97, which has been approximated to -2.0, at θ = 0.02). Markers D9S1780, D9S1785, D9S1867, D9S922 gave exclusion at θ = 0 and D9S1867 gave a value of Z_{max} = -2.0 at θ = 0.01. Markers D9S264 and D9S167 again gave negative lod score values with D9S167 which gave Z_{max} = -2.11 at θ = 0.03.

Haplotype analysis of the D9S153 - D9S152 interval

The D9S153-D9S152 interval was reduced between D9S1867 and D9S264 Généthon markers which are spanning a 4 cM region. Individuals III-2, IV-2, IV- 3, IV-24 and IV-29 cut off the D9S153 marker from the region. Individuals III-4, IV-8, IV-10, IV-12 cut off the D9S152 and D9S264 markers. The marker which seems to be shared by the majority of the patients was the D9S1867 marker. Nevertheless the individual IV-29 appeared to recombine at the D9S1867 marker level, sharing only the region of D9S264 and D9S152 of the disease chromosome. In addition, apparently healthy carriers of the haplotype of the affected relatives were individuals III-5, IV-8, IV-17 at the D9S1867 marker level. Finally, IV-24 and V-24 individuals, defined as "unknown" on the clinical point of view, shared the seek haplotype with the exception for the D9S153 marker's allele.

In conclusion, the refinement of the FDC locus led to an interval between the two new flanking markers D9S1867 and D9S264 of 4 cM, with respect to the previously determined 15 cM interval between D9S153 and D9S152. Within this shorter interval, a double recombinant (IV-14), another recombinant patient (IV-29), and 3 healthy carriers were identified. These results, along with the two point lod score data, appeared to highly reduce the probability of mapping the disease locus within this region.

Given these negative results, attention was given again to another region of chromosome 9, which in the whole genome approach was found to give a suggestive lod score value, and, for these reasons had been originally included in the "hot" spot regions. Attention had not been turned on this region since the beginning of the study since the polymorphic markers mapping in this region were not highly informative. This region is encompassed by the D9S285-D9S157 interval.

Analysis of the D9S285-D9S157 interval

As mentioned above, the D9S285-D9S157 interval was the first identified region of chromosome 9 which gave a lod score value suggestive of linkage even according to more recently published criteria [Lander and Kruglyak, 1995] at locus D9S156 (>1.9 at θ 0).

In this interval, only two new polymorphic markers became available for a suitable refinement of the map: D9S1782 and D9S1839 colocalizing with D9S156 in the new Généthon genetic linkage map (Dib *et al.*, 1996). The two point lod score calculation for D9S1782 and D9S1839, showed exclusion value at $\theta = 0$ and lack of informativeness at higher fraction of recombination; nevertheless the lod scores were positive for both markers with a $Z_{max} = 1.65$ and 0.72 respectively at $\theta = 0.08$. These markers were not used for the haplotype analysis for their low number of alleles in FDC1 family and for the high rate of homozygousity.

Therefore, the haplotype analysis at this interval was performed with markers D9S285, D9S156 and D9S157. It showed a perfect homogeneity in the inheritance of the candidate chromosome in the affected family members. The complete chromosome segment was inherited by all the patients except IV-14 individual who shows invariably the healthy chromosome segment from the affected father. Interestingly the chromosome inherited from the

healthy mother III-3, had the same pattern as the one inherited from the father and linked to the disease. Again, 3 healthy carriers (III-5, IV-8 and IV-17) sharing the same haplotype were found. These results are in patent contrast with a high likelyhood of linkage of the FDC gene in this region.

Screening of the whole chromosome 9

A chromosome 9 complete scan was considered to be needed since the ultimate human genetic linkage map had been published (Dib *et al.*, 1996). Therefore, other markers were tested along the entire chromosome 9 in order to better define the family haplotype **(Fig. 13)**.

Thirty additional microsatellite markers were used to better cover the complete available chromosome 9 linkage map. A complete panel of these markers is listed in **Table 6**. For all of them the two-point lod score calculation was performed. Unfortunately, none of them resulted to indicate exclusion or non significant values (most of them were poorly informative).

Enlargement of the number of family members and haplotype analysis with markers belonging to the "hot regions"

Given the negative or non definitely convincing results obtained in the above mentioned studies, and in order to obtain a definitive prove of the possible role of chromosome 9-encoded genes in the disease, a final effort has been recently performed by further enlarging the number of analyzed family members. Additionally, a further clinical follow up has been conducted on the previously analyzed individuals.

A new expedition to the place of residence of the FDC1 family (in Calabria, Southern Italy) was thus organized. An overall number of 120 family members was investigated and historical reconstruction of the family till the end of the 18th century was performed. More than one hundred new blood specimens were sampled and isolation of lymphocytes, establishment of

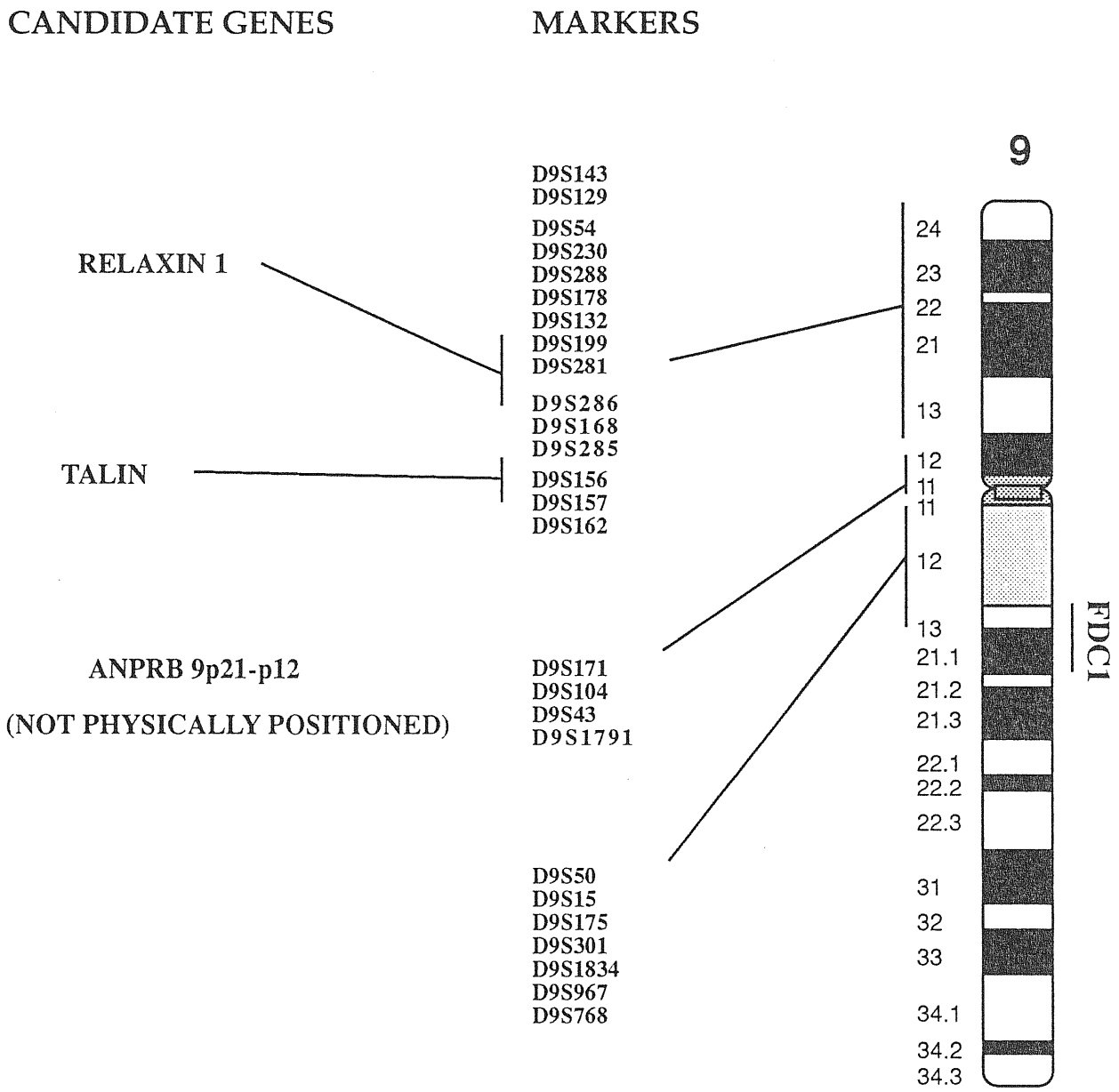


Figure 13. Refinement of the genetic linkage map of chromosome 9 with about 30 additional highly polymorphic markers.

D9S 143 (9p-ter), D9S 129 (9p-ter)
D9S 54
D9S 230, D9S 178, D9S 288
D9S 132
D9S 281
D9S 1676, D9S 286
D9S 168
D9S 285 (9p22.3) a 14.332 Mb dal 9pter
D9S 1839 (9p22.3)
TALIN
D9S 156 (9p22.3) a 14.929 Mb dal 9pter
D9S 1782 (9p22.3) a 15.189 Mb dal 9pter
D9S 157 (9p22.3) a 16.169 Mb dal 9pter
D9S 162 (9p22.2) a 17.035 Mb dal 9pter
D9S 1846
D9S 171 (9p21.3) a 20.657 Mb dal 9pter
D9S 104 (9p21.3) a 24.172 Mb dal 9pter
D9S 43 (9p21.2) a 25.554 Mb dal 9pter
D9S 50 (9p12)
D9S 1791 (9p11.1) a 48.430 Mb dal 9pter
Centromere a 51.000 Mb dal 9pter
D9S 202 (9q12) a 57.197 Mb dal 9pter
FRDA (9q12) a 57.232 Mb dal 9pter
D9S 15 (9q12) a 57.597 Mb dal 9pter
D9S 166 (9q12) a 59.531 Mb dal 9pter
ANX1 (9q12)
D9S 301 (9q12) a 20.657 Mb dal 9pter
D9S 175 (9q21.12) a 74.695 Mb dal 9pter
D9S 1834 (9q21.32) a 75.180 Mb dal 9pter
D9S 967 (9q21.33)
D9S 768 (9q22.1)
D9S 153 (9q22.1) a 92.559 Mb dal 9pter
D9S 1780 (9q22.1) a 92.808 Mb dal 9pter
D9S 1867 (9q22.1) a 92.931 Mb dal 9pter
D9S 244 (9q22.1)
D9S 1843 (9q22.1)
D9S 922 (9q22.1) a 93.427 Mb dal 9pter
D9S 264 (9q22.1) a 93.735 Mb dal 9pter
FRA9D
D9S 167 (9q22.1) a 93.911 Mb dal 9pter
D9S 152 (9q22.1) a 94.216 Mb dal 9pter
D9S 303

Table 6. List of the polymorphic markers used for the refinement of the chromosome 9 map. Where known, the cytogenetic banding, the position of already mapped genes and the relative distance from the telomere, in the SHRG-RH map are indicated.

B-lymphoblastoid cell lines and DNA extraction from total blood was performed.

As an initial approach to these new samples, two new family branches were selected for their potential informativity, since they were accounting for 27 new members, which were not available before. These new 27 individuals plus additional 9 family members belonging to already studied family branches, were amplified for markers D9S285, D9S156, D9S157, D9S153, D9S1867 and D9S264. Furthermore, crossover analysis was done by haplotype construction **(Fig. 14)**.

Regarding the D9S153-D9S264 tested interval, the affected members IV-3 and IV-4 showed a recombination which excluded the D9S153 marker.
The patient IV-15 still inherits the healthy chromosome that leads us to suppose this affected individual to be a double recombinant.
Patients IV-11, IV-13 and IV-36 were confirmed to be recombinant in this interval. Furthermore, individuals III-7, IV-9, IV-21, IV-23 and IV-31 were identified or confirmed as healthy individuals carrying the candidate haplotype at the D9S153 and D9S1867 interval.

As far as the D9S285-D9S157 interval is concerned, all but one patients, including the new affected III-16, shared the same haplotype. Only individual IV-15 was an obligate recombinant, but, interestingly, he inherited a haplotype identical to the candidate one from the mother III-3.
Again, healthy carriers for the whole region were identified in individuals IV-9, IV-35, IV-26, and IV-31, while individual IV-18 was recombinant within this region at the D9S156 locus level.

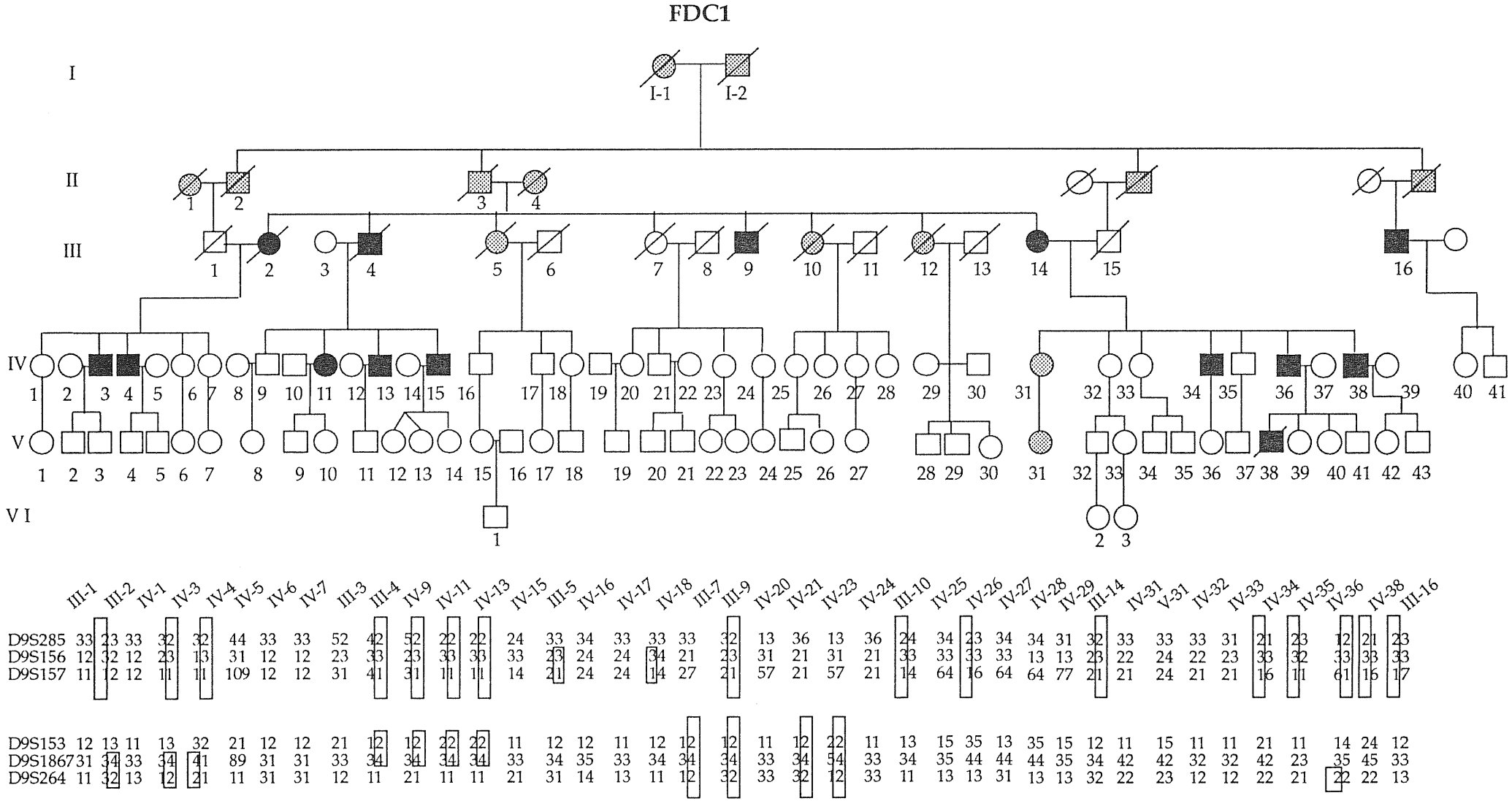


Figure 14. Pedigree of the key members of the FDC1 family after the last enlargement. Individuals are indicated by generation and pedigree number. Affected status is indicated by solid symbols, unaffected status is indicated by clear symbols and unknown status is indicated by stippled symbols. Haplotype analysis for markers mapping in the "hot spot" regions.Pedigree of the key members of FDC1 family after the last expedition to the place of residence of the family.

Conclusions

The overall survey of the results obtained by the above mentioned studies on chromosome 9 inheritance in the FDC1 family currently leads to disappointing conclusions. Linkage was excluded for the D9S153-D9S264 interval (linkage in this regions would require the assumption of 7 different recombination events - 1 double and 5 single - in a 4 cM region). Linkage is formally excluded also for region D9S285-D9S157, unless a clearly affected individual carrying a "healthy" haplotype is considered as a phenocopy and 8 healthy individuals are misclassified due to the low penetrance of the disease.

It appears evident that there are some limiting factors in the study. First of all, due to the structure of the family (one of the largest reported in the literature), with at least two known loops, and for the genetic characteristics of the study population, the informativness of the markers was lower than reported. Secondly, the low penetrance in the low-age group, leading to the exclusion from the study of members younger than 20 years, greatly reduced the study subjects of the last generations. Moreover, due to the severity of the cardiac pathology, several elderly family member were dead, further reducing the informativity of the pedigree.

RESULTS-PART II

MOLECULAR GENETICS OF FDC FORMS WITH MUSCULAR INVOLVEMENT

X-LINKED DILATED CARDIOMYOPATHY

XLDC was linked to dystrophin (DMD) gene (Muntoni *et al.*, 1993; Towbin *et al.*, 1993; Yoshida *et al.*, 1993). In 2 families with DC, no-male to male transmission and mild increase of serum CK, the mutation analysis of the dystrophin gene was performed.

Family XLDC1

In this family, 4 family members were examined: 2 affected brothers (II-1, II-2) one healthy brother (II-3) and the healthy mother (I-1). The proband (II-1), a 24 years old male, was admitted to the Cardiology Department in Trieste, Italy, for a severe heart failure developed within the last 6 months. The diagnosis was idiopathic dilated cardiomyopathy. He was completely free of any clinical and laboratory sign of skeletal muscle disease, including increased serum creatine kinase (CK) level. Additionally, he had been a competitive basketball player for several years. He died two years later for post-surgical complications after heart transplantation. After several years, his brother was examined because of atypical chest pain and found to have a severely dilated and hypokinetic left ventricle. The levels of the MM isoform of creatine kinase (MM-CK) were moderately increased (1.5 - 3 times the normal values), without clinical evidence for muscle disease and with normal electromyography, despite some reported episodes of urine pigmentation after physical exercise and sporadic myalgias. These observations prompted us for a

careful examination of the other family members. The pedigree of this family is reported in **Figure 15, panel A-I.** The proband's mother (I-1) and the second brother (II-3) were found to be normal at physical examination, electrocardiography and echocardiography. Individual II-4 died at birth for unknown causes and individual I-2 died for a non-cardiac disease. The reported history of first- and second-degree relatives was completely negative.

Haplotype analysis

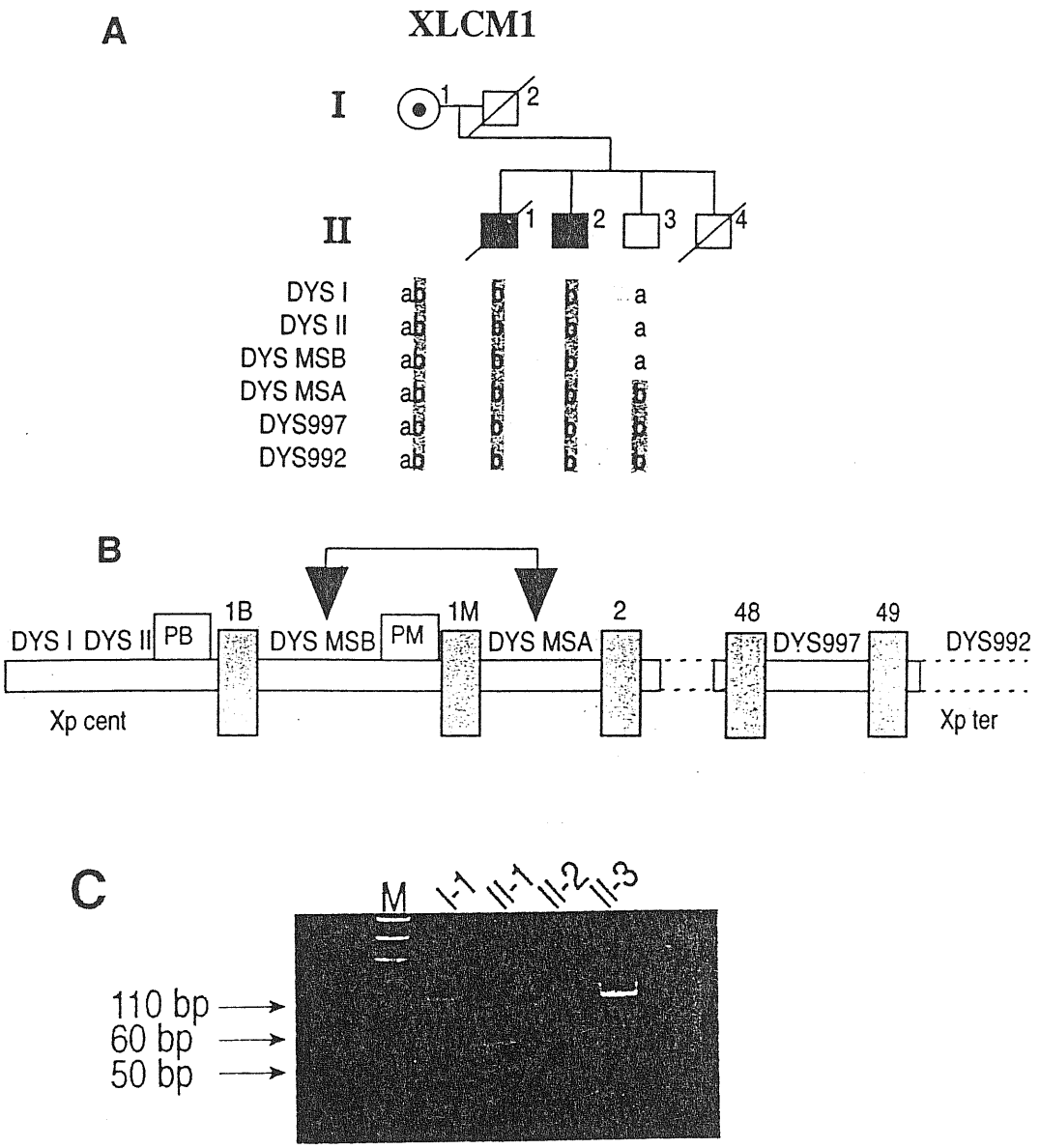
The increase in serum CK in individual II-2 led us to examine the pattern of inheritance of the dystrophin gene in this family, by means of highly polymorphic microsatellite markers located within, or very close to the gene. This analysis demonstrated segregation of the same allele in the two affected brothers, and the occurrence of a critical intragenic recombination in individual II-3, occurring in the interval between DYS MSB and DYS MSA (**Fig. 15, panel A-II, panel B**). Due to this recombination, the chromosomal region downstream of marker DYS MSA (telomeric to the muscle promoter) was shared by the unaffected (II-3) and the affected (II-1 and II-2) brothers, while the portion upstream of the marker DYS MSB (centromeric to the muscle promoter) was recombined in the healthy brother. Because of this finding, we focused our attention on the analysis of the 5' end of the dystrophin gene, including the muscle promoter and first muscle exon.

Mutational analysis

The screening of the DMD gene performed using multiplex PCR (Beggs *et al.*, 1990; Chamberlain *et al.*, 1988a), and the amplifications of the first muscle exon, muscle and brain promoter and second exon failed to show any deletion.

A point mutation screening was then undertaken. Sequential segments of the region including the muscle promoter, the first muscle exon and 250 bp

Figure 15. Panel A: I, Pedigree of XLDC1 family; II, haplotype of the XLDC1 family members. Panel B: Dystrophin gene regulatory elements and the polymorphic markers studied for the haplotype analysis. Panel C: restriction analysis of PCR products from XLDC1 individuals. A new restriction site for *MseI* was created by the G->T mutation.



of the first intron (Klamut *et al.*, 1990) were amplified by PCR and screened for single strand conformation polymorphism (SSCP). Individuals I-1, II-1, II-2 and II-3 were studied. The PCR products encompassing the second half of the first exon and the beginning of the first intron (nucleotides 215-324) had a different migration pattern. In individuals II-1 and II-2, two prominent bands were detectable, which were clearly different from those found in individual II-3. All four species were detected in the mother (I-1; **Fig. 16, panel B**).

The nucleotide sequence of a 1387 bp region encompassing the muscle promoter, the first muscle exon and the first portion (250 bp) of intron 1 was determined. As predicted by SSCP analysis, the only mutation found was a G to T transversion at the 3' first muscle exon-intron boundary in the genomic DNA from individual II-2. This mutation occurs at the first base of the GT dinucleotide consensus sequence of the 5' splice site, which is absolutely conserved in all mammalian intron sequences (**Fig. 16, panel C**). The unaffected brother II-3 showed a normal sequence.

The G to T mutation detected in individual II-2 introduced a new restriction site for *Mse* I. As expected, the restriction analysis of a PCR product encompassing the mutation site generated a single undigested band (of 110 bp) from the unaffected individual II-3, and two digested bands (of 60 and 50 bp respectively) from the two affected brothers II-1 and II-2, showing complete segregation of the mutation with the disease. Accordingly, three bands corresponding to the wild type and to the mutated alleles, were detected after restriction of the PCR product generated by the amplification of the genomic DNA from the mother **(Fig. 15, panel C)**.

Family XLDC2

The proband presented with severe heart failure and DC at the age of 18. He had a family history compatible with X-linked inheritance: a maternal cousin, a maternal uncle and an obligate female carrier were dead of DC at the

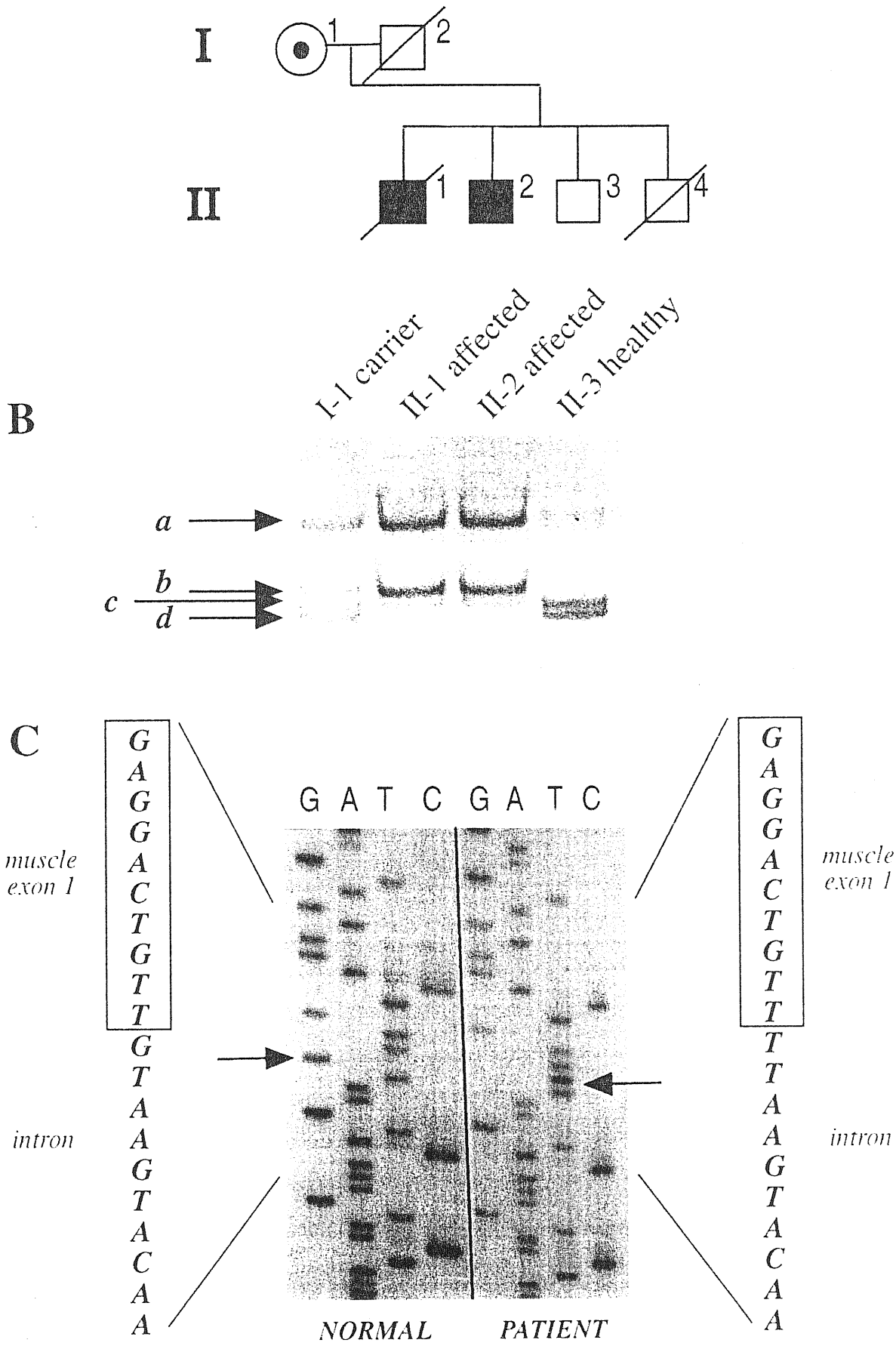

A
XLDC1
I
1
2
II
1
2
3
4
B
I-1 carrier
II-1 affected
II-2 affected
II-3 healthy
a
c
b
d
C
G A T C G A T C
muscle exon 1
intron
G A G G A C T G T T
G T A A G T A C A A
muscle exon 1
intron
G A G G A C T G T T
T T A A G T A C A A
NORMAL
PATIENT

Figure 16. Panel A: Pedigree of XLDC1 family. Panel B: SSCP analysis of PCR products encompassing the first muscle exon-intron junction. Individual ssDNA strands are indicated by arrows (a-d) Individuals II-1 and II-2 (affected) carry the same dystrophin allele, that is different form the one of individual II-3 (healthy). The mother (I-1) is heterozygous as expected. Panel C: Sequence analysis of the first muscle exon-intron of dystrophin gene. In the patient II-2 a G->T point mutation abolishes the 5' splice site consensus sequence. The normal control is represented by the healthy broher (II-3).

age of 42, 36 and 57, respectively. Interestingly, another maternal cousin had Duchenne muscular dystrophy and died at the age of 22; all other affected family members did not showed overt signs of skeletal muscle involvement. The proband had a serum CK level 2 to 3 times normal.

Multiplex PCR analysis.

Multiplex PCR analysis was performed in order to identify deletions of the coding region of the DMD. A deletion of exons 48 and 49 was found in the proband III-1 **(Fig. 17)**.

Expression of dystrophin in the heart and in the skeletal muscle

Analysis of the histological and immunohistochemical features, and of dystrophin expression was performed in left ventricular endomyocardial and skeletal muscle biopsies obtained from individual XLDC1-II2.

Histological examination of the skeletal muscle biopsy showed a mild variability in fiber size with the presence of mildly scattered atrophic fibers, rare splittings, and no increase in interstitial connective tissue. The number of internal nuclei was slightly increased. At immunocytochemistry with antibodies directed toward the N-terminus, mid-rod, and C-terminus of dystrophin, all fibers appeared continuously labelled, but the intensity of fluorescent labelling was paler than in control muscle **(Fig. 18, panel B and C)**.

The endomyocardial biopsy showed a severe fibrosis and a marked variability of fiber size. Contrary to the pattern detected in the skeletal muscle, no immunoreactivity was found with antibodies directed toward the N-terminus and mid-rod region of dystrophin, while only a weak reactivity at the periphery of cardiomyocytes was observed using a C-terminal anti-dystrophin antibody **(Fig. 18, panel E)**.

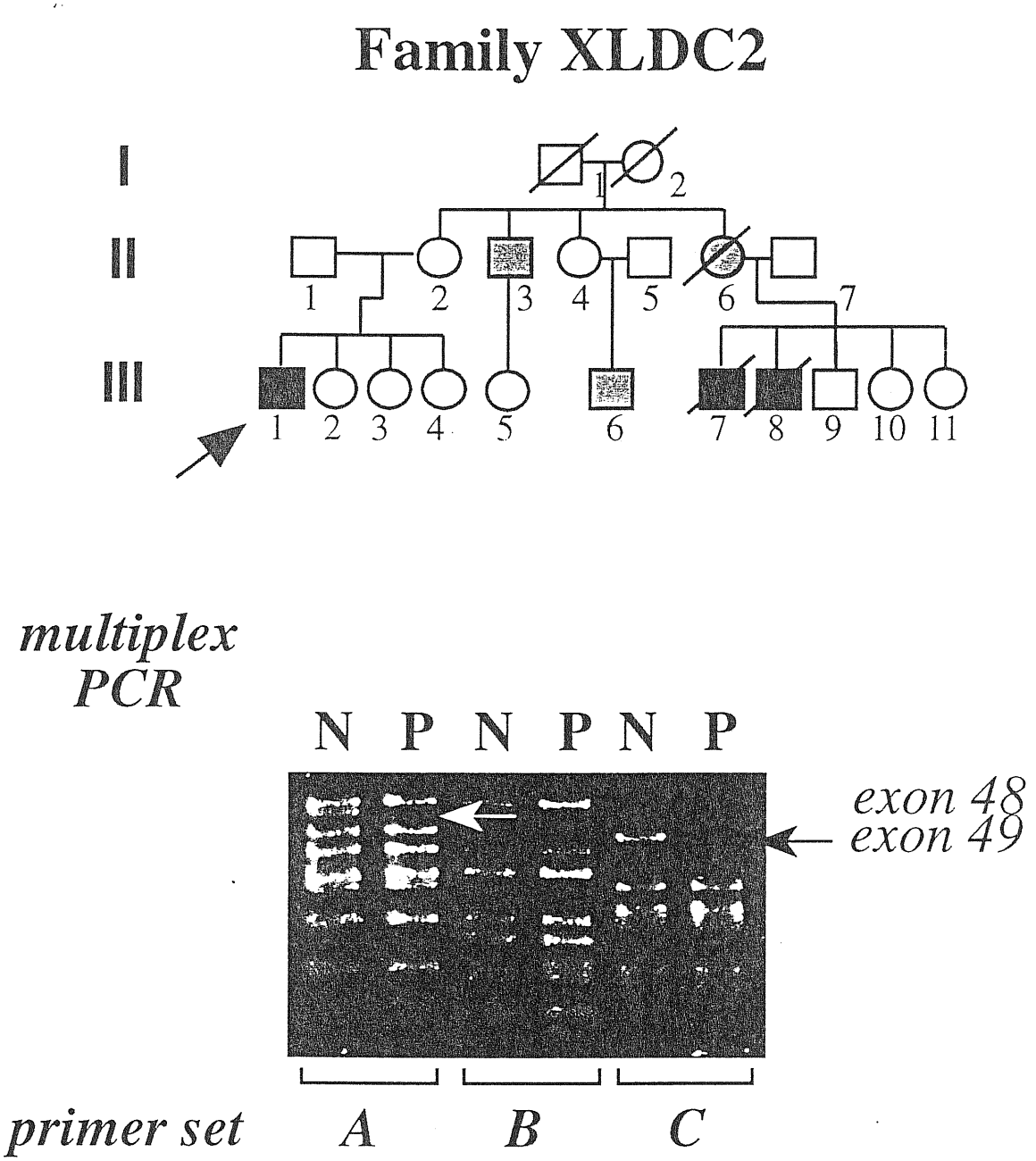
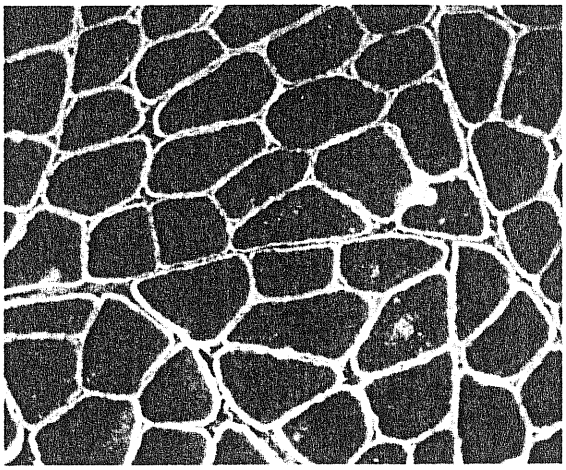
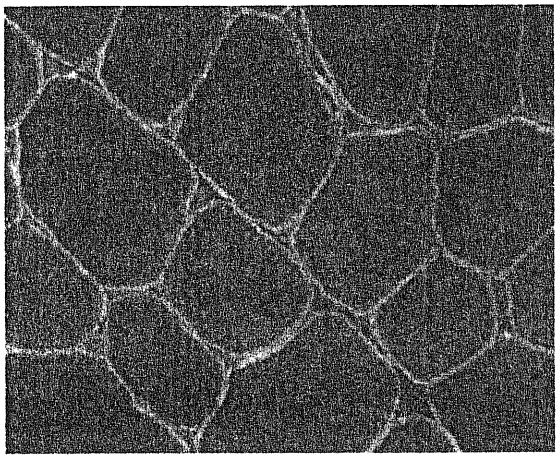


Figure 17. Pedigree of family XLDC2. The proband presented with IDC and mild increase of CK. The proband (arrow) had five relatives with diagnosis of dilated cardiomyopathy (bleck squares) or suspected IDC (gray circle), or "muscle dystrophy" (gray squares). Multiplex PCR demonstrated the deletion of exon 48 (set A) and 49 (set C) in the proband (lanes P) compared to a narmal control (N).

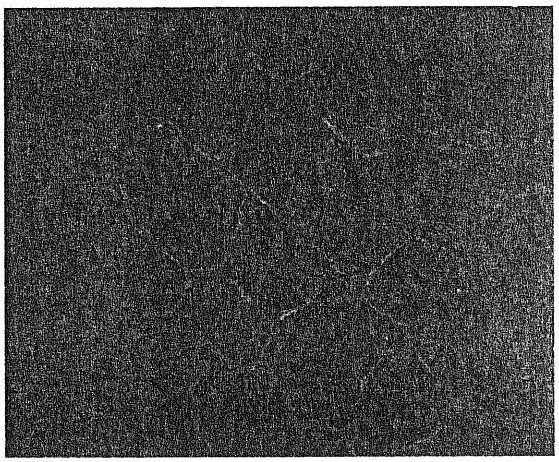
A



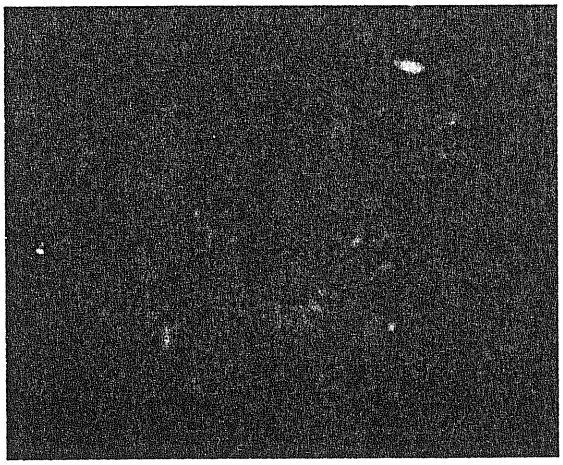
B



C



D



E

Figure 18. Immunocytochemistry of skeletal muscle. Cryostat sections of skeletal muscle from a normal individial (A), were immmunolabelled with antibodies to C-ter of dystrophin. The cryostat sections of the patient's skeletal muscle were labelled with antibodies against both N-ter (B) and C-ter (C). These antibodies show reduced but structurally preserved staining of the muscle of the patient as compared to that of the normal control (x 180). The section of the cardiac muscle from the patient immunostained with antibodies to the N-ter (D) shows complete absence of dystrophin (x250). Only a week reactivity could be detected with antibodies against the C-ter of the protein (E) (x 250).

The analysis of expression of the dystrophin mRNA was performed on total RNA isolated from skeletal and endomyocardial biopsies, and reverse transcribed using random hexanucleotide primers. PCR amplification of this cDNA was performed using a muscle isoform-specific forward primer and a reverse primer located in exon 2 **(Fig. 19, panel A)**. No cDNA corresponding to the muscle isoform could be amplified either from the muscular or the cardiac tissues of the patient. Additionally, no PCR products of larger size (suggestive of the usage of alternative cryptic splice sites) were obtained, even after extended PCR cycling (not shown). When forward primers specific for the brain-and the Purkinje cell- dystrophin mRNA isoforms were used, clear amplification products were observed with the cDNA obtained from the muscle of the patient, but not from the heart **(Fig. 19, panel B)**. When primers specific for the Dp71 transcript were used (Hugnot *et al.*, 1992), amplification was obtained for the cDNAs from the heart samples from both the control and the patient. The presence of the Dp71 mRNA (encoded by the 3' region of the dystrophin gene (Hugnot *et al.*, 1992; Lederfein *et al.*, 1993), is consistent with the weak reactivity detected in the heart sample of the patients with antibodies directed exclusively against the C-terminal of the protein.

AUTOSOMAL DILATED CARDIOMYOPATHY WITH SKELETAL MUSCLE INVOLVEMENT (MDDC)

Clinical and genetic features

In our family study, 3 families presented DC associated with subclinical skeletal muscle involvement resembling XLDC, but with *autosomal dominant* pattern of transmission (8% of the families with FDC). An inconstant and mild increase of the serum creatine kinase level (max. 3.6 time the normal values) was found in 5 subjects, belonging to 2 families, and varied from clearly pathological level to normal in the same subjects. The

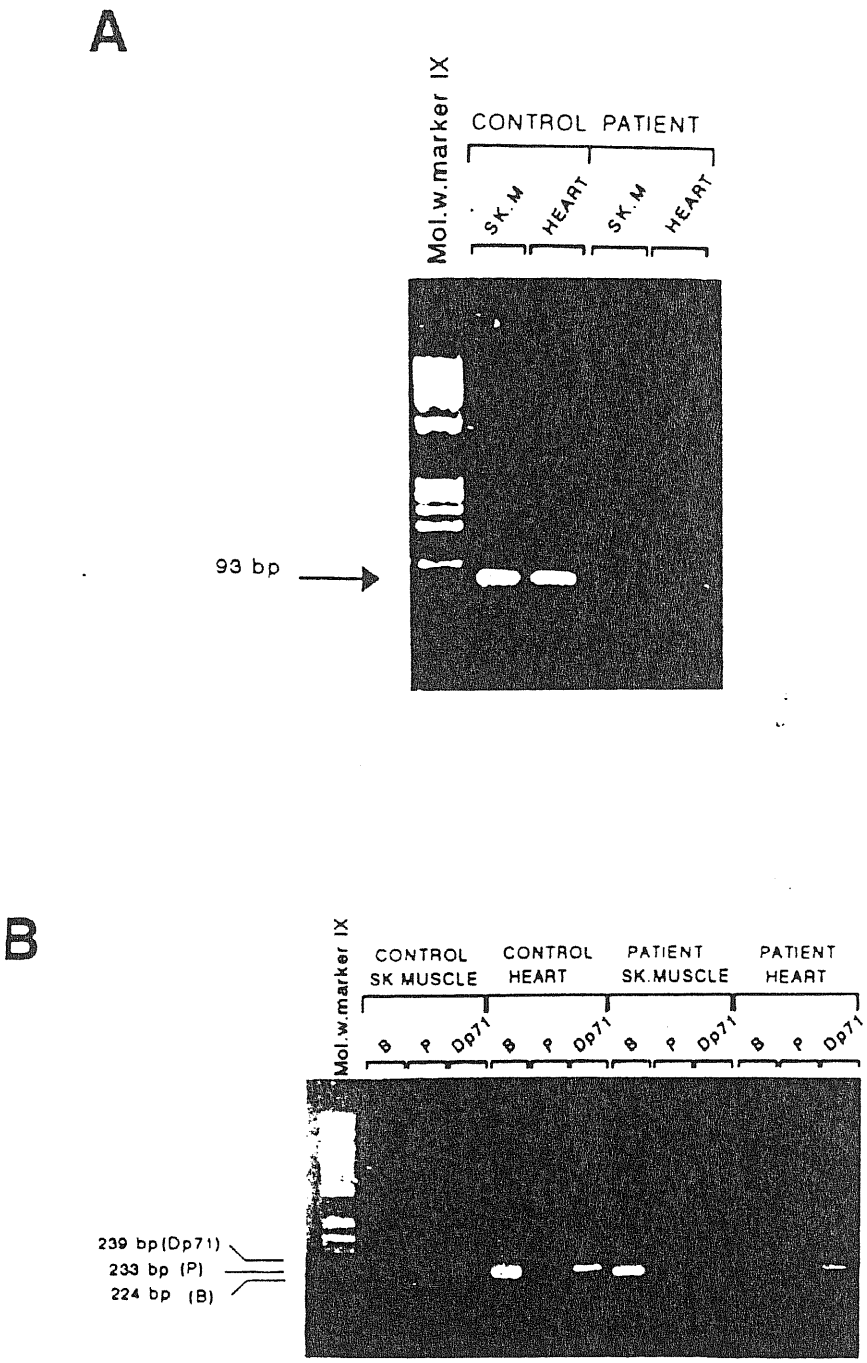


Figure 19. Panel A: amplification of the muscle isoform of dystrophin. The 93 bp band corresponding to the amplification of the muscle isoform was obtained in normal skeletal and cardiac muscles, but not in the corresponding tissues from the II-2 patient. Panel B: amplification of the brain (B), Purkinje (P) and Dp71 dystrophin isoforms from skeletal and heart muscle of a normal individual and of patient II-2. In the comtrol individual, transcritpion of the brain isofrom could be detected in the heart, while it was completely absent in the heart of the patient. On the contrary, both the brain and Purkinje-cells transcripts could be obtained from the skeletal muscle of the patient. In both the patient's and the control's hearts, amplification from the Dp71 cDNA could be obtained.

clinical characteristics of the disease did not differ from that found in other forms of DC.

The family pedigree were constructed, and two to three generations were studied **(see Fig. 6)**. Of the 22 studied subjects, 8 were affected, 10 were unaffected, and the remaining, who did not fulfil the criteria for the diagnosis of DC but presented minor clinical, ECG or echocardiographic abnormalities, were defined as "unknown".

The molecular genetic studies were performed on the largest, therefore the most informative MDDC family available, named MDDC1.

In this family, 12 members were studied. The affected family members (4 cases) had a typical DC. The presence of ventricular arrhythmia and conduction defects required permanent pacing in 2 cases. The serum CK level ranged from normal to increased (x3 times normal). Electromyographic examinations were consistent with an intrinsic disease. Even if there were no overt signs of skeletal muscle involvement, in patient III-5 a careful neuromuscular examination could suggest a mild form of Limb-girdle muscular dystrophy. The young affected relatives showed an initial myocardial involvement and the presence of mild signs of skeletal muscular dystrophy.

Histologic and immunocytochemical studies

Endomyocardial biopsy was performed in 6 patients, showing pathological but aspecific changes in all cases, such as hypertrophy and atrophy of myocells, interstitial fibrosis and infiltration of lymphocytes and monocytes. In 2 cases, however, histological signs of myocarditis were present, active in one (Aretz, 1986), while healed in the other. A rich component of fibrosis was also clearly evident. Five members of the families underwent skeletal muscle biopsy. Muscle was characterised by variability in the fibre dimensions for the presence of atrophic and hypertrophic fibres in the same

sample of tissue; moreover the number of nuclei was increased and a slight fibrosis was present. In some samples the intramyofibrillar pattern was abnormal with the aspect of minicore. Normal was the distribution of dystrophin, DAG and desmin; normal was also cytochrome oxidase evaluation.

The last family presented more evident changes: the skeletal muscle morphology was clearly abnormal and characterised by non-specific myopathic changes: variability in fibre size due to the presence of atrophic (<20 μm) and hypertrophic fibres (>90 μm), fibre splitting and increase in internal nuclei. One patient showed secondary signs of dystrophinopathy in skeletal as well as in heart muscle (reduced immunoreactivity with C-terminal antibodies). The same patient showed reduced expression of beta dystroglycan and laminin beta 1. The other patients did not show reduced expression of any of the studied membrane proteins.

Positional candidate approach

Thirteen members of the three generation family MDDC1 were investigated by linkage analysis, whereas the remaining two families were excluded because not enough informative.

Linkage analysis was performed using polymorphic markers chosen for their intragenic or very close localisation to the gene of interest. A total of 32 dinucleotide polymorphisms (microsatellites) were analysed. This polymorphic markers were linked or very closed to genes coding for cytoskeleton and extracellular matrix proteins, as already derscibed above.

Unfortunately, none of these loci appeared to be linked to MDDC, indicating once more the heterogeneity and the complexity of molecular genetic studies of DC.

DISCUSSION

About three decades ago, cardiomyopathies were first identified and defined as heart muscle diseases of unknown etiology by the WHO/ISFC. The most frequent form was estimated to be dilated cardiomyopathy (DC) (Codd *et al.*, 1989), which was associated by a high mortality and morbidity, therefore representing a relevant clinical problem. Moreover, DC represents the leading indication for heart transplantation in the word. As a consequence of the clinical relevance, the identification of the cause of the disease was considered a research priority (Manolio *et al.*, 1992).

Important advances for the understanding of the etiology of DC have been made by clinical genetic and molecular genetic studies. Firstly, a systematic screening of families of DC patients has recently demonstrated that genetic factors, previously considered to be very rare, have a major role in the pathogenesis of the disease. The occurrence of a genetic transmission (familial DC or FDC) in our controlled study was detectable in about half of patients with DC, with a prevalent autosomal dominant trait. These data could still underestimate the real frequency of FDC, due to the possibility of missing affected individuals, particularly when the pedigrees are small, to the reduced compliance of the families when the history of disease is not evident, to the absence of early markers of disease, and to the reduced penetrance. In FDC the penetrance is age-related, therefore the disease can be clinically not apparent, in particular in the young family members. Furthermore, the risk of disease was estimated to be approximately 20% in first degree relatives of DC patients (Keeling *et al.*, 1995) and, in absence of a clinical examination, in our patient population, the risk of missing a familial trait was about 20% .

According to other authors (Keeling *et al.*, 1995; Michels *et al.*, 1992), we could not identify any clinical parameter able to differentiate the familial form. On the other hand, studies on the clinical and genetic characteristics of

the inherited form clearly indicate the existence of genetic heterrogeneity. Several different subtypes have been observed in our study, leading to a new classification: autosomal dominant FDC (AD-FDC), autosomal recessive FDC (AR-FDC), conduction defects with later development of dilated cardiomyopathy (CDDC), X-linked dilated cardiomyopathy (XLDC), subclinical muscle disease with dilated cardiomyopathy (MDDC) and, finally, unclassifiable dilated cardiomyopathy.

However, the most important advances in this field are due to the introduction of a molecular genetic approach to the study of FDC, which has recently started to produce important results toward the identification of the disease genes and the pathogenesis of dilated cardiomyopathy.

PART I-AUTOSOMAL DOMINANT FORM OF DILATED CARDIOMYOPATHY

The most frequent form of FDC is the autosomal dominant form (AD-FDC) which occurs in the 55% of the families in our patient population.

To identify the disease gene of AD-FDC and avoid the risk of genetic heterogeneity, a single large Italian kindred with typical isolated dilated cardiomyopathy and dominant mode of inheritance was selected. This family pedigree was intensively studied either for candidate gene loci or for a genome-scan approach. After excluding more than 95% of the whole genome, an indication for linkage was recognised on chromosome 9. A first indication of linkage came from the detection of a *suggestive*, although not sufficiently significant association (Lander and Kruglyak, 1995) with the DS9156 marker.

After a complete screening of the entire chromosome, the region of higher probability was localised at the D9S153-D9S152 interval level, postulating a double recombinant (IV-14). The possibility of a double recombination was supported by the map distances available at that time.

Including in the study two other AD-FDC families with identical clinical characteristics, a cumulative lod score value of 3.69 was obtained with the polymorphic marker D9S153, and the FDC locus was localized in the D9S153-D9S152 interval ranging from 7 up to 15 cM, according to the data available from different published human genetic maps (CHLC, 1994; Gyapay *et al.*, 1994).

Errors and inconsistencies have been recorded in all genetic linkage maps with a low density of microsatellite markers. This is one reason that led us to enrich the human framework map with additional genetic markers. In fact, the last Généthon human genetic linkage map contains about 6000 polymorphic microsatellites with integrated physical mapping data (Dib *et al.*, 1996), thus ensuring a much higher accuracy than in the past.

Studies on systemic disorders associated with dilated cardiomyopathy indicate that genes encoding for proteins with different functions can lead to the same phenotype of myocardial dilatation and dysfunction (Dec and Fuster, 1994; Kelly and Strauss, 1994). This observations has suggested to investigate on several genes mapping within this region.

Candidate gene approach

Once the linkage was established, a candidate genes approach was undertaken. Among the genes considered for further studies, the most promising gene appeared to be the tropomodulin gene (TMOD). Human tropomodulin is a 40.6-kD tropomyosin-binding protein from the human erythrocyte membrane skeleton that binds to one end of erythrocyte tropomyosin and blocks the head-to-tail association of tropomyosin molecules along actin filaments; TMOD is localised at or near the free (pointed) ends of the thin filaments and this localisation is not dependent on the presence of myosin thick filaments (Fowler, 1996).

Tropomodulin may, therefore, regulate the length and/or the organisation of actin filaments by differential binding to tropomyosin isoforms (Sung and Lin, 1994).

The tropomodulin gene has been assigned to human chromosome 9q22.2-q22.3, a region that is also known to contain several other genes and disease loci and is proximal to the loci for gelsolin and alpha-fodrin. The gene for tropomodulin is expressed in major human tissues at different levels in the following order: heart and skeletal muscle, much greater than that in brain, lung, and pancreas, which is greater than that in placenta, liver, and kidney (Sung *et al.*, 1996).

In this study, the cDNA of the TMOD gene was reverse transcribed and amplified from total RNA extracted from lymphoblastoid cell lines of two patients. TMOD gene has been completly sequenced without finding any mutation, also in comparison to the sequence of a healthy member.

Apart from the tropomodulin gene, other suitable genes were also: i) the annexin I gene (ANX1), encoding for proteins which form ion channels in a phospholipase-dependent manner; ii) the protein kinase, cAMP-dependent, catalytic, gamma subunit (PRKACG) gene, coding for a protein which regulates the calcium channel ion conductance in the heart (Catterall, 1988). PRKACG has been mapped very closed to the FRDA gene (Montermini *et al.*, 1995). In fact, PRKACG is laying on the centromeric side of the F8101 marker which is flanking a 150 kb segment containing the FRDA gene (Campuzano *et al.*, 1996); iii) the gene for the Friedreich Ataxia (FRDA or frataxin), which is a severe neurodegenerative disorder with autosomal recessive transmission frequently associated with hypertrophic cardiomyopathy, (Campuzano *et al.*, 1996; Carvajal *et al.*, 1996). FRDA has been mapped at 9q13-q21.1 (Chamberlain *et al.*, 1988b; Fujita *et al.*, 1989; Hanauer *et al.*, 1990). The study of intragenic polymorphic markers permitted to exclude all the tested genes as possible candidates for causing FDC. The mixed technical tools as sequencing and

linkage study, used to search any association between the genes and the disorder, were utilised because of the lack of information, for all those genes, or the complete cDNA or whole gene sequences. Furthermore, the use of further microsatellites allowed us to test new available genetic markers since the characterisation of those genes and the region in which they were lying was attained by independent researchers.

Unfortunately, all the markers used were not helpful in cutting the region, also because most of them although mapped on cytogenetic maps have not been used at that time for building up radiation hybrid maps integrated with genetic linkage maps. This led to the fact that for almost all of them, the cytogenetic chromosome banding was the only site reference. Moreover the localisation of the possible candidate genes mapped in OMIM, was always referred to a cytogenetic position which for a linkage map refinement study has a very relative value.

All these issues recall the dramatic need, stressed by the Human Genome Project Organisation, of a really suitable integrated map as a framework for all the genetic studies.

In fact, the markers position differs depending on the RH (Radiation Hybrid) maps used to physically localise the microsatellite. The maps currently used are the WIT (Withehead Institute of Technology) and the SHGRC (Sanger Human Genome Research Centre). Finally, although the physical and genetic maps show some discrepancies, the most accurate human linkage map which has some of its markers physically mapped in all RH maps, appears to be the last Genethon release (Dib *et al.*, 1996). So, the Genethon linkage map was the reference map for all my further studies including the haplotype analysis. The degree of discrepancy between different physical maps rendered impossible any further precise localisation of the Genethon markers as well as other sources markers.

Linkage refinement of the FDC region

Since the study of candidate genes did not produced a positive result, a refinement of the FDC locus utilising the new genetic markers which became available from different sources was considered necessary. On the other hand, the 15 cM interval was considered to be too large to be screened by a positional cloning approach within a feasible period of time using the current procedures.

The new microsatellites used for the refinement did not provided further evidence of linkage. On the contrary, with a more detailed definition of the region using genetic markers within the FDC region, new recombinations were identified: one occurred in an affected family member (IV-29). Moreover, the affected individual IV-14 was confirmed obligate double recombinant, although the size of the region was reduced to a total of 6 cM. The distance between the markers D9S1867and D9S264, considered new borders of the FDC locus, was of 4 cM, according to the latest edition of the Généthon human genetic linkage integrated map (Dib *et al.*, 1996).

The presence of this patient which shows on the whole genetic linkage study on chromosome 9 the inheritance of the healthy genotype, represents a point of criticism for the entire molecular genetic approach. It could be supposed that this patient underwent a *de novo* mutation causing a phenocopy. Although it could explain the healthy haplotype, it seems to be quite unlikely to happen, since the rarity of such an event.

Two-point lod score analysis confirmed the findings of the haplotype analysis, and the lod score value appeared to be negative or non informative for the new markers D9S967, D9S1867 and D9S264; for none of the other markers a significative lod score could be detected. This change in the results of two-point linkage analysis is explained by the increase in the nuber of recombinants and healthy carriers. While the presence of healthy carriers can be assumed due to the reduced penetrance which characterises the disease, the

presence of two patients who do not share the same allele as the other affected members, reduced the probability of the previously identified linkage.

Refinement of the whole chromosome 9 linkage study

Even if the region identified on the long arm of chromosome 9 appeared less likely to be the region containing the disease gene, the haplotipe analysis still indicated the tendency of chromosome 9 to be coninherited with the disease within this family. Therefore, a further effort was done to investigate the region 9p22-p23 flanking the D9S156 genetic marker. In the initial screening of chromosome 9, this microsatellite was the first showing a lod score value of "suggestive linkage", according to the recent linkage analysis criteria of Lander and Kruglyak (Lander and Kruglyak, 1995). However, the relative low informativeness of the family at the D9S156 marker level, did not allow conclusive results at that time.

A detailed linkage study of the p arm of chromosome 9 appeared to be necessary, since a large panel of new genetic markers became available from several genetic maps.

As expected, the data obtained from the previous study were confirmed and the absence of a stronger statistical result was probably due to the insufficient family size and its structure with consequent low informativeness.

In order to improve the information obtainable from FDC1 family, one of the largest ever described in the literature with this pathology, a new scientific expedition to the family living place was planned in August 1997, with the purpose of expanding the family size and confirm the previously assessed phenotypes. In the large number of new examined individuals, two new affected individuals were identified.

Haplotype analysis of the enlarged size of the FDC1

The larger size of the family was supposed to be wide enough to give those informations which could not be possible to obtain from a smaller number of individuals. More than 30 new family members were studied for the following markers: D9S285, D9S156, D9S157, D9S153, D9S1867, D9S264.

This map refinement has detected new recombination events, occurring at the patients level, which drop down the informativity of the FDC locus identified by the D9S153-D9S152 markers interval. The haplotype analysis confirmed that the previously identified FDC locus cannot be considered anymore likely to contain the FDC gene.

Another possible candidate locus was represented by the D9S285-D9S157 interval. In fact, this region on the p arm of chromosome 9, showed a better concordance of the haplotypes of the patients. Actually, during the genome-scan for the genetic mapping of the AD-FDC locus, the first result suggestive of linkage to chromosome 9 was obtained by analyzing the microsatellite marker D9S156. The statistical data that led us to shift to the 9q as a more probable region, were due to the family pedigree size, the intrinsic informativeness of the tested genetic markers and a possible overestimation of the penetrance of the family itself.

Unfortunately, also analysis of this genomic region did not produce positive and definitive results.

Conclusions

The overall survey of the results obtained by the above mentioned studies on chromosome 9 inheritance in the FDC1 family currently leads to disappointing conclusions. Linkage was excluded for the D9S153-D9S264 interval (linkage in this regions would require the assumption of 7 different

recombination events - 1 double and 5 single - in a 4 cM region). Linkage is formally excluded also for region D9S285-D9S157, unless a clearly affected individual carrying a "healthy" haplotype is considered as a phenocopy and 8 healthy individuals are misclassified due to the low penetrance of the disease.

It appears evident that there are some limiting factors in the study. First of all, due to the structure of the family, with at least two known loops, and for the genetic characteristics of the study population, the informativness of the markers was lower than reported. Secondly, the low penetrance in the low-age group, leading to the exclusion from the study of members younger than 20 years, greatly reduced the study subjects of the last generations. Moreover, due to the severity of the cardiac pathology, several elderly family member were dead, further reducing the informativity of the pedigree.

In addition to these specific problems, some constrains which limit the efficacy of a linkage analysis approach to the disease are intrinsic to the disease itself. These constrains are particularly due to the low and highly variable penetrance of the disease, the absence of early clinical markers which hampers definitive clinical diagnosis, and the premature mortality.

Given these considerations, it is auspicable that novel large kindreds with genetic transmission of the disease will be rendered available in the future for a hopefully more easy and successful genetic analysis.

PART II-FDC FORMS WITH MUSCULAR INVOLVEMENT

X-linked Dilated Cardiomyopathy

The only known gene causing dilated cardiomyopathy is dystrophin genes causing X-linked dilated cardiomyopathy (XLDC). XLDC is a familial heart disease presenting in young males as a rapidly progressive congestive heart failure, without clinical signs of skeletal myopathy (Muntoni *et al.*, 1993; Towbin and Ortiz-Lopez, 1994). All the studies that were addressed to the discovering of the gene defects leading to XLDC, put in evidence the importance of the 5' end involvement of the dystrophin gene (Nakamura *et al.*, 1997; Towbin and Ortiz-Lopez, 1994).

The dystrophin gene is the largest gene so far identified in humans, covering more than 2.5 Mbp and containing 79 exons (Tinsley *et al.*, 1994). The encoded product, dystrophin, is a 427-kd cytoskeletal protein has a critical role in the membrane stability, the force transduction and the organisation of membrane specialisation in skeletal and cardiac myocytes (Ahn and Kunkel, 1993).

Besides dystrophin, FDC has been also associated with the defect of the cytoskeletal protein metavinculin due to a defect in alternative mRNA splicing (Maeda *et al.*, 1997).

XLCM phenotype is associated to mutations at the 5' end of the muscular isoform of the dystrophin gene

To date, six mutations in the DMD gene have been reported for a clinical phenotype of dystrophinopathy characterised by a severe dilated cardiomyopathy without apparent myopathy. The first was a deletion that removed the muscle promoter, the first exon and part of the first muscle

intron at the 5' end of the DMD gene (Muntoni *et al.*, 1993). Other deletions were recorded to be associated to XLDC phenotype and involving the muscular promoter region (Franz *et al.*, 1995; Oldfors *et al.*, 1994; Towbin and Ortiz-Lopez, 1994).

In the work presented in this thesis, we have identified a first point mutation of the dystrophin gene, associated to XLDC. This point mutation has found to abolish the splicing site of the first muscular exon (Milasin *et al.*, 1996).

The discrepant phenotype of the patients which shows an apparent normal skeletal muscle and a selective heart involvement, is consistent with the normal distribution of dystrophin in skeletal muscle biopsy. In fact, only its quantitative reduction could be suggestive of a dystrophinopathy, in contrast to the absolute absence of the protein in the heart. The production of the dystrophin mRNA at the muscle level, is sustained by transcription from the brain and Purkinje mRNA. Thus, it is likely that the absence of the muscle dystrophin isoform leads to a compensatory expression pattern which utilises the other tissue-specific isoform promoters to prevent the myopathy in the affected males (Milasin *et al.*, 1996).

Recently, other investigators have identified a missense mutation in exon 9 at nucleotide 1043 which causes an alanine to threonine substitution at the highly conserved aminoacid position 279. This mutation leads to a dramatic change of the polarity at the level of a evolutionary conserved region of the protein causing the substation of a beta-sheet domain to an alpha-helix which destabilizes the protein (Ortiz-Lopez *et al.*, 1997).

Multiplex PCR in XLDC

The need of characterising DMD, BMD and XLDC patients by the identification of mutations occurring at the exons level of DMD gene, has led to set up a diagnostic procedure based on the contemporary amplification of

panels of primers which give specific exons as PCR products. By multiplex PCR analysis, we have characterised two patients with XLDC phenotype showing a deletion of the 48-49 exon of the dystrophin gene, usually associated to a Becker muscular dystrophy phenotype (Muntoni *et al.*, 1997). Usually mutations at the 48-49 exon level were associated to BMD patients with a severe muscle involvement (Melacini *et al.*, 1993).

A possible explanation to the fact that these deletions occur in XLDC patients could be the presence, within the intron 48, of sequences relevant to the function of dystrophin in the cardiac muscle. This intronic sequence would be either removed or preserved by an intragenic deletion involving exon 48, according to the intron 48 breakpoint, while any deletion involving both exon 48 and 49 would remove these sequences. Another explanation could be that exon 49 encodes for a critical domain involved in the heart function (Muntoni *et al.*, 1997).

Meng et al., 1996 demonstrated an association of dystrophin with Z-disc region of cardiac muscle but not with analogous Z-lines of skeletal muscle. Furthermore, dystrophin localizes to the transverses tubules of cardiac but not skeletal muscle. These differences could account for the different phenotype of dystrophin mutations in the heart and in the muscle. Obviously, further investigations about the molecular basis of these differences are required.

Furthermore, different defects within different critical functional regions of the dystrophin protein, or defects in the regulation of the expression of the dystrophin mRNA could lead to the phenotype of X-linked dilated cardiomyopathy.

The potential mechanism for the cardiospecificity of dystrophin mutations may be due to different regulative patterns between cardiac and skeletal muscle (Beggs, 1997; Klamut *et al.*, 1996; Milasin *et al.*, 1996). The expression study of the dystrophin gene has showed, besides the tissue specific promoters, further regulative elements. Remarkably, the muscle intron 1 has

been shown to contain an enhancer sequence which is functionally regulated by a MEF-1/E box and a MEF-2/AT-rich sequence motifs (Klamut *et al.*, 1997). The presence of this muscle specific enhancer containing such regulative sequences, indicates a regulative mechanism very similar to those identified in other muscle specific genes studied to date (Klamut *et al.*, 1997).

To date, many efforts have been carried on to understand the regulative pattern of the DMD gene, which apart from being the largest human gene, it appears to undergo complex pattern of either up or down regulation (Klamut *et al.*, 1990; Klamut *et al.*, 1989; Lev *et al.*, 1987; Tennyson *et al.*, 1995); (Nakamura *et al.*, 1997); (Klamut *et al.*, 1997)]. Nevetheless, the regulation of the dystrophin gene still remains unclear especially regarding the differences of expression of the DMD isoforms investigated at the skeletal and heart muscles level (Klamut *et al.*, 1997; Nakamura *et al.* , 1997; Gramolini and Jasmin, 1997).

Autosomal Dominant Form of Dilated Cardiomyopathy with skeletal involvement

Considering the high level of clinical and genetic heterogeneity recognised for the DC, the identification of a new form of dilated cardiomyopathy as the MDDC, appears to be a natural even though not trivial consequence of the improvements in understanding more towards the characteristics of the DC phenotypes and the genetic factors implied in its development.

The important point to focus is the fact that the only previously reported form of DC with signs of skeletal muscle involvement was the X-linked DC which is caused by deletions or point mutations at the level of the

muscle promoter and first muscle exon/intron region of the dystrophin gene (Milasin *et al.*, 1996; Muntoni *et al.*, 1995b).

The main difference between the MDDC and the other DC forms is the autosomal dominant mode of inheritance and the involvement of the skeletal muscle which has an impaired function which varies in severity from patient to patient. This observation led us to think that other proteins of similar function or cellular localisation could be involved.

By analogy with dystrophin, other cytoskeletal proteins appear to be potential candidates for causing dilated cardiomyopathy. For example, we have observed a patient with DC associated to signs of muscle dystrophy deficiency of the sarcoglycan alpha (adhalin) protein, which is one of the DAG complex proteins (Fadic *et al.* 1996). The absence of transcription of metavinculin (the cardiac isoform of vinculin) has been shown in patient with DC (Maeda *et al.*, 1997).

This speculation was at the basis of an effort which should test all the already identified loci throughout the genome, which contained important genes for the skeletal and cardiac function or genes identified by linkage analysis for those disorders which were striking also the cardiac or the skeletal muscle. Unfortunately, the screening of more than 30 loci was unable to link any of them to MDDC. Nevertheless, the hypothesis that a structural protein, as it occurs at the XLCM level, could be the responsible for the MDDC is still likely.

APPENDIX

STRATEGIES FOR HUMAN GENE IDENTIFICATION

The genetic inheritance of human disorders has been identified to be caused by DNA sequence variation already seventeen years ago and the identification of genes responsible for simple mendelian traits has become a straightforward procedure (Botstein *et al.*, 1980). The different strategies used to map human disease genes have undergone continuous improvement in the last years, also by taking advantage of automation (Reed *et al.*, 1994; Schuler *et al.*, 1996) as well as from new technical approaches like whole genome radiation hybrid mapping (McCarthy, 1996).

Generally the choice of strategy for identifying a disease gene, depends on what kind of resources are available (i.e.: animal models, chromosomal abnormalities, clone libraries, etc.) and on the known knowledge about the pathogenesis of the disease. Whatever the approach, the ultimate aim is to identify a certain number of candidate genes which then have to be tested individually for evidence that implicates them as the disease locus. The evidence that a candidate gene is likely to be the disease locus can be achieved by various means. A good candidate gene should have an expression pattern consistent with the disease phenotype, although usually only a subset of expressing tissues are involved in the pathology.

The most popular method is the screening for patient-specific point mutations in the candidate gene, because it could be generally applicable and comparatively rapid. Identifying mutations in most of the affected individuals strongly suggests that the disease gene has been identified. However, formal proof requires additional evidence

Positional cloning.

The identification of possible genes from a contig of genomic clones of a certain sub chromosomal region is called positional cloning (Collins, 1995). Acting the positional cloning can be extremely laborious, and is usually not seriously attempted until genetic or physical mapping has located the disease to within about 1-2 Mb of DNA. For some disorders, such fine mapping is not practicable. If a disorder is rare, it may be particularly difficult to identify crossovers that narrow the location of the disease gene to a small interval, and the chance of identifying a patient with a suitable disease-associated cytogenetic abnormality which could refine the map location, may be virtually zero. In such case candidate gene approaches are the only way forward.

Nevertheless, the most fruitful first step is generally mapping the disease to a specific sub chromosomal localization although, oftenly the initial localization defines a relatively large candidate region of 10 Mb or more. Such initial locations may come from various sources. One of these is the loss of heterozygousity screening which identifies a chromosomal region which is commonly deleted in tumors. Tumor suppressor genes important in cancer are often first located this way (Fountain *et al.*, 1992).

Analogous characteristics has the study of chromosomal abnormalities which can implicate a general region in a disease. For example, translocations and deletions identified Xp21 as the translocation of the Duchenne Muscular Dystrophy (DMD) gene (Kunkel *et al.*, 1985). Still, the most common means of localization for inherited disorders is the linkage analysis.

In vitro and in vivo models.

Subsequently to the identification of a disease gene, there could be the need of studying the pathophysiological mechanisms of the disease with an *in vivo* model. Sometimes, disorders caused by loss of function mutations have

a reversible phenotype. If a cell line displaying the mutant phenotype is available, it would be possible to restore the normal phenotype by complementing the genetic deficiency by the transfection of a cloned normal allele into the cultured disease cells [reviewed in: "An introduction to Genetic Analysis", Griffiths A. et al., 1996 (Freeman eds.)].

An example of the application of animal models tool in the study of primary and secondary cardiac diseases, is the mouse model for mitochondrial myopathy and cardiomyopathy created generating a knockout mice deficient in the heart/muscle isoform of the adenine nucleotide translocator (Ant1). The Ant1 gene encodes for a carrier protein which is involved in the electrochemical gradient utilized by mitochondria to phosphorylate ADP to ATP and therefore to store oxidative energy as a useful source for the metabolism of the cell (Brown and Wallace, 1994). The knockout mice Ant1^{-}, a part from a severe myopathy, shows also an hypertrophic cardiomyopathy (Graham *et al.*, 1997). Another knockout mouse has been recently generated to resemble a primary myocardial defect. This mouse represents the first model for dilated cardiomyopathy and heart failure phenotype (Arber *et al.*, 1997). In this mouse, the striated muscle-specific Lim-only protein MLP (muscle LIM protein) which leads to myogenic differentiation associated with the actin-based cytoskeleton (Arber *et al.*, 1994; Arber and Caroni, 1996] was disrupted. The phenotype of these mice shows disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy and heart failure (Arber *et al.*, 1997).

THE HUMAN GENOME PROJECT

The Human Genome Project is an international project whose ultimate aim is to obtain a complete description of the human genome by DNA

sequencing. It started after a workshop held in Alta, Utah, in December 1984. The project was supported partially by the US Department of Energy (DOE) and was basically intended to evaluate the current and future advance in all technologies devoted to improve the genomics.

One of the major aims was considered the generation of high resolution genetic and physical maps of the human genome. Among the remarkable benefits of such big effort, there will be the chromosomal localization of genes on the genetic map. From the medical point of view, placing precisely a gene and other closed genetic markers enables the researchers to perform the linkage analysis in family pedigrees with inherited disease.

The first human genetic map comprehensive of 403 polymorphic loci, was published in 1987 and was mostly based on RFLP markers (Donis-Keller *et al.*, 1987). This was a huge work and although it represented a dramatic improvement in human genome mapping, some serious drawbacks remained such as the average spacing between the markers (>10 cM), which was still considerable. Moreover, a major limitation was the reliance on RFLP markers, which are not very informative and very difficult to type.

Other DNA polymorphisms were subsequently used to construct human genetic maps. The major advances have been done by using the microsatellite markers. They are very abundant and dispersed almost uniformly throughout the entire genome; besides they are highly informative and easy to type. By focusing the efforts on this type of markers, researchers at the Genethon laboratory in France were able to provide a second-generation linkage map of the human genome (Weissenbach *et al.*, 1992). They used suitably polymorphic CA/TG repeats, mapping them to specific chromosomes by typing panels of human-rodent somatic cell hybrids and performing statistical linkage analysis on markers from individual chromosomes. A total

of 813 markers, of which 605 showed a heterozygousity above 0.7, were organized into 23 linkage groups.

In 1994 a new human genetic linkage map become available; it was containing 2,066 (CA)n short tandem repeats, 60% of which was showing a heterozygousity of over 0.7 (Gyapay *et al.*, 1994).

Finally, two years later, the Genethon published their ultimate human genetic linkage map comprising (CA)n tandem repeats. Actually, this last version of the Genethon human linkage map consists of 5,264 short tandem (AC/TG)n repeat polymorphisms with a mean heterozygousity of 70%. The map spans a sex-averaged genetic distance of 3,699 cM and comprises 2,335 positions, of which 2,032 could be ordered with an odds ratio of at least 1,000:1 against alternative orders. The average interval size is 1.6 cM; 59% of the map is covered by intervals of 2 cM at most and 1% remains in intervals above 10 cM (Dib *et al.*, 1996).

Alternative maps have been produced by other laboratories with the occurrence of discrepancy between data (CHLC, 1994; Gyapay *et al.*, 1994). This is due to two major reasons: firstly, not all genetic maps have used the same sets of reference families, so differences in the allele frequencies among different populations occur. Secondly, different genetic markers have been used to construct different genetic maps and the linkage relationship between markers in different genetic maps may not be known.

Due to these problems, the collaboration within the international research community has became more important. In fact, the problem of reference families has been solved by distributing the same set of family to over 100 independent laboratories by the CEPH (Centre d'Etudes du Polymorphisme Humaine). Further, the study of the linkage relationship between markers used in different genetic maps has provided an integrated map. Finally, it is perceived that the latest genetic map released by Genethon

can provide the suitable framework for constructing a high resolution physical map of the human genome (Dib *et al.*, 1996; Schuler *et al.*, 1996).

A variety of different types of physical maps are possible using advanced technical tools. For example, by FISH (Fluorescence In Situ Hybridization) (Heiskanen *et al.*, 1996; Trask, 1991), is possible to achieve a high resolution cytogenetic mapping. Further maps have been obtained by mapping natural chromosome breakpoints using somatic cell hybrid panels containing fragments derived form translocation and deletion chromosome or by mapping artificial breakpoints using radiation hybrid (RH) (Stewart *et al.*, 1997; Walter *et al.*, 1994). This technique has been developed by a previous irradiation and fusion gene transfer procedure (Westerveld and Freeke, 1971). Using the fusion of a human cell line with a recipient rodent cell line, it was possible to rescue genomic DNA fragments from a lethal irradiation of the diploid human cell line (Goss and Harris, 1975). In 1985, this technique led to the gene mapping of the X chromosome (Willard *et al.*, 1985). However, the first high resolution map constructed using the radiation hybrids was done for chromosome 21 (Cox *et al.*, 1990). This map has been done using hybrids generated by Irradiation Fusion Gene Transfer (IFGT) between a donor somatic cell hybrid containing a single human chromosome and the recipient rodent cell line. The first Whole Genome-Radiation Hybrid map (WG-RH) instead was made using the Goss and Harris approach which resulted to be the most feasible for the wide human genome (Walter *et al.*, 1994).

However, the major goal for a WG-RH is to generate genetically anchored YAC (Yeast Artificial Chromosome) and cosmid contigs across the human genome. It could be done mapping RHs to chromosome cosmid and YAC libraries by direct hybridization of Alu PCR product of individual hybrids to library clones, gridded on nitrocellulose membrane (Monaco *et al.*, 1991). For sure, one of the most important applications of RHs was to use them as framework map of microsatellite markers onto which was possible to

map several ESTs (Expression Sequence Tags) along the whole human genome (Gyapay *et al.*, 1996). This framework map was generated with LOD scores of grater then nine, with the ESTs placed using pairwise linkage procedure with a maximal LOD score of grater than ten (Gyapay *et al.*, 1996). So far, several RH maps have been constructed (Hudson *et al.*, 1995; McCarthy, 1996). These maps usually vary each other by the amount of irradiation of the human diploid cell line; in order to have a high resolution map a certain amount of radiation, measured in centi Rad or cR (the radiation unit used) is given to the human diploid cell line.

Also the clone contig map represents a comprehensive collection of fragments covering the whole genome. Generally these collections are made by overlapping inserts cloned in special vectors like YACs (Yeast Artificial Chromosomes), BACs (Bacterial Artificial Chromosomes), bacteriophage l, P1, PACs (P1 Artificial Chromosomes) and cosmids (fig.). The first generation physical map of the human genome was constructed by exhaustive screening of the CEPH YAC library which contains 33000 YACs with an average insert size of 0.9 Mb, representing 10 haploid genome equivalents (Cohen *et al.*, 1993).

In order to map an increasing number of human genes, an EST (Expressed Sequence Tag) library was also developed. This requires to sequence the cDNAs from a specific human tissues cDNA clone library, and then to map them back to other physical maps.

THE LINKAGE ANALYSIS

Principles and applications.

Linkage analysis is a technical tool in genetic investigation and its principles could be applied to all the sexually reproducing diploid organisms

(Conneally and Rivas, 1980). The goal that has to be achieved is to discover how often two loci are separated by meiotic recombinations. In other words, the aim of every human linkage mapping is to find which genetic markers are physically linked to the disease locus in order to undergo the lowest recombination rate among the patients of a family pedigree (Ott, 1985). The recombination fraction is a measure of the genetic distance so, if the two loci are on different chromosomes they will segregate independently with a 0.50 of recombination fraction which corresponds to the 50% of probability for both the alleles of the two loci to be inherited.

Two loci which lie very closed together on a chromosome, rarely will be separate by recombination, because only a crossover located precisely in the small space between the two loci will create recombinants.

Furthermore, once the linkage has been confirmed, it is useful for analyzing sets of alleles on the same small chromosomal segment which tend to be transmitted as a block trough a pedigree. Such a block of alleles is known as a haplotype. The haplotype defines recognizable chromosomal segments which can be tracked through pedigrees and through populations.

The haplotype analysis is normally used to examine individual cross-overs in the pedigree, in order to define the closest distal and proximal markes flanking the disease gene. If the haplotype does not reveal any recombination, haplotypes can be treated for mapping purposes as alleles at a single highly polymorphic locus.

Furthermore, the greater is the distance between two loci, the more likely it is that a crossover will separate them. Thus, the recombination fraction defines the genetic distance between two loci. However, this is not the same as the physical distance. The recombination fraction of 0.01 (or 1%) is defined as showing a distance between two loci of 1 centimorgan (1 cM) apart on a genetic map. However, for distances more than 5 cM, human genetic map distances do not simple define the recombination fraction between pairs

of loci. Loci which are 40 cM far from each other on a genetic map will show rather less than 40% recombination because the recombination fractions never exceed 0.5, however far apart the loci are.

The mathematical relationship between recombination fraction and genetic map distance is described by the mapping function. A mapping function converts the raw data of the recombination fraction into a genetic map. The simplest map function, Haldane's function assumes that crossovers occur at random along a chromatid pairs in prophase of meiosis I and have no influence on one other.

Obviously, crossovers are not occurring randomly, but there are "hot-spots" across the genome at which crossovers occur at different rates, as it has been shown for human chromosome 9 (Povey, 1992). In fact, when a crossover occurs and a chiasma is formed, the formation of a second chiasma nearby is inhibited due to a phenomenon called interference (Attwood *et al.*, 1994).

Interference is intensively studied in Drosophila and yeast, and has recently been demonstrated occurring in man (Schmitt *et al.*, 1994). Several mapping functions have been set up to create a mathematical model of this phenomenon, allowing the calculation also of varying degrees of interference, and it is not clear which is the most appropriate for human mapping.

Among all mapping functions, the one which is most widely used is the Kosambi's function (Ott, 1991; Terwilliger and Ott, 1994).

In a human cell, 49 is the average number of crossovers which have been showed by chiasma counts to occur during male meiosis (Morton *et al.*, 1982). The total male genetic map length implied by chiasma counts, is of 2450 cM, since each crossover gives 50% recombinants. If we add all together the chromosome lengths, the best estimate from linkage mapping is of 2644 cM (Gyapay *et al.*, 1994).

However, in female meiosis the rate of recombinations is higher than in male meiosis, and it accounts for a total genome map length of 4481 cM (Gyapay *et al.*, 1994). So, across the 3000 Mb of the human genome, 1 male cM averages 1.13 Mb and 1 female cM averages 0.67 Mb with a sex average value of 1 cM = 0.9 Mb. Furthermore, the actual correspondence varies widely for different chromosomal regions. The most extreme deviation occurs at the pseudoautosomal region at the tip of the short arms of the X and Y chromosomes. The Y chromosome has an obligatory crossover within 2.6 Mb region, so that it is 50 cM long. Thus, for this region in males 1 Mb = 19 cM, whereas in females 1 Mb = 2.7 cM. Generally, the recombination rate is higher towards the telomeres of chromosomes and less towards the centromeres, especially in males [reviewed in: "Human Molecular Genetics", Tom Strachan and Andrew P. Read, 1996; Bios eds.].

Human Y chromosome has the unique feature of having no genetic map because it is not subject to synapses and crossing overs in normal meiosis except the pseudoautosomal region (Ellis and Goodfellow, 1989; Cooke. 1985). Instead, X chromosome of course undergoes normal recombination in females, and can genetically mapped in female meiosis.

A part from these issues, in order to map human disease genes, performing a linkage analysis, which is a disease-marker mapping procedure, is a compulsory step. Since genetic markers are mendelian characters which are polymorphic enough to show a high degree of heterozygousity, they are considered to be useful means to study family pedigrees for human disorders.

Linkage analysis is a quite demanding research effort to carry on. Therefore, to start such a big project, some major points have to be taken in account. In fact, before selecting a family pedigree to perform the genetic mapping, the disorder to study should be not particularly rare so that it will be

possible to increase the number of studied members for any further map refinement. Moreover, the family is required to have a good structure for mapping, with a reasonable hope that family members will not all be homozygous (and hence uninformative) for the marker.

It is very difficult to collect suitable family sample for mapping. Moreover the informativness of each marker is a key requirement. Although ten meiosis are sufficient to give evidence of linkage if there are not recombinants, 85 meioses would be needed to give equally strong evidence of linkage if the recombination fraction was 0.3. Because the difficulty in sampling enough family materials, mapping requires markers spaced at intervals no greater than about 20 cM across the genome even though a more dense map will be highly desirable such as 10 or 5 cM spaced map (Lander and Kruglyak, 1995). This means that the minimum number of markers required for a complete genome scan to map a disease gene will be around 200. Moreover, what is needed is not only each marker informativeness, but more fundamentally informative meioses. Generally, a meiosis is not informative for given marker if the subject is homozygous for the marker, and also in half of the cases where the subject is heterozygous but the spouse has the same heterozygous genotype. In order to have a high rate of informative meiosis, highly heterozygous markers with high number of alleles are needed.

The standard tools for linkage analysis are now microsatellites. However, dinucleotide repeat sequences undergo replication slippage during PCR amplification so that each allele gives a little ladder of "stutter bands" on a acrylamide gel. This can make the results hard to read. On the contrary, tri- and tetranucleotide repeats usually give clearer results with a single band from each allele, and so these are gradually replacing dinucleotide repeats as the markers of choice. The automation technologies applied to molecular genetic studies, have led to the development of compatible sets of microsatellite markers which can be amplified together into multiplex PCR

reactions, and have allele sizes which allow them to be run in the same gel lane without producing overlapping bands. Furthermore, the automated genome mapping has started to replace conventional auto radiographic procedures for analyzing amplified microsatellites since the major source of error is in the reading of auto radiographs (Beckman *et al.*, 1993). Nowadays, the genome mapping technology largely prefers the use of fluorescence labeling in several colors for automated gels (Reed *et al.*, 1994).

Statistical constraints in linkage analysis.

From the statistical point of view, the major tool in linkage analysis is the two point mapping. In order to quantify the probability that a certain marker and the disease locus are linked, a Bayesan calculation has to be performed: this shows that the conventional p = 0.05 threshold of significance for the chi-squared (χ^2) test requires 1000:1 odds from the linkage analysis. A computer-based calculation of this probability, introduces another statistical entity: the lod score.

Lod score, Z, first defined by Morton (1955), represents the logarithm of the odds (probability) that the loci are linked (with recombination fraction θ) rather than unlinked (recombination fraction 0.5). Being a function of the recombination fraction, lod scores are calculated for a range of θ values and the maximum value Z estimated. The value Z (x) is referred to as a two-point lod score, as it involves linkage between only two loci (i.e.: a disease locus and a marker locus). If a mapper is investigating a set of families, the overall probability of linkage in a set of families is the product of the probabilities in each individual family, therefore lod scores (being logarithms) can be added up across families.

Lod scores are calculated by looking at each meiosis in turn and comparing the likelihood of the observed genotypes on the alternative hypotheses of linkage or no linkage. In order to do this, is very important that

each meiosis would be phase-known which means that for all those meiosis we know the parents genotypes.

Nevertheless, using computer programs it is possible to account the all possible phases of family members although they are not unambiguously defined. The best known programs are LIPED (Ott, 1974) and MLINK which are part of a package called LINKAGE (Lathrop *et al.*, 1984). Both of these can be run on a personal computer (Terwilliger and Ott, 1994).

Although the use of computer analysis led to an enormous simplification of the work of the investigators, it is useful to have a good background in statistical genetics since a lot of diseases show a more complex human traits which render much complicate the data to elaborate and the parameters to set up (Lander and Kruglyak, 1995). In fact, during a genome scan, there are oftenly positive results occurring just by chance. Besides, the choice of suitable parameters to drop down the relative costs of false positive and false negatives is not trivial (Lander and Kruglyak, 1995).

A very important point to take in to account is the level of significance of the data either for the pointwise significance level (nominal probability that one would encounter a striking deviation at a specific locus just by chance) or for the genome-wide significance level (Lander and Kruglyak, 1995). Historically, the P value, which reflects the pointwise chance of observing a deviation as high as P (x) under independent assortment, has been considered to be significant when it reaches the P = 0.05 value.

Actually, in a whole genome scan, the expected number of chromosomal regions which show a linkage statistics exceeding a threshold T is given by a Poisson distribution. According to this mathematical theory, at least 24 times a region showing a P = 0.05 value will occur by chance across the genome (Lander and Botstein, 1989; Feingold *et al.*, 1993). A P = 0.01 will occur 7-8 times; P = 0.001 slightly more than once; P = 0.0001 about 0.2 times and P =

0.00002 about 0.05 times. This means that there is a 5% chance to find randomly a region with a P value bordering on 2×10^{-5}.

If we want to keep the chance of encountering a false positive at no more than 5%, it is required to impose a threshold of $Z \geq 4.1$, lod ≥ 3.6 or $P = 2 \times 10^{-5}$ (Lander and Kruglyak, 1995). On the contrary, using less stringent criteria, there will always be a substantial chance (more than 5%) of reporting false linkages.

The traditional general criteria for linkage and exclusion residing on the lod score values, indicates that all the lod scores are zero at $\theta = 0.5$ since they are measuring the ratio of two identical probabilities, and log 10 (1) = 0. For smaller θ values, the critical thresholds for a single test are $Z = 3.0$ and $Z = -2.0$. In fact, $Z = 3.0$ or 1000:1 odds is the threshold for generally accepting linkage with a 5% chance of error. Instead, linkage can be rejected if $Z \leq -2.0$. Values of Z between -2 and +3 are inconclusive. If there are not recombinants, lod score will be maximum at $\theta = 0$. If there are recombinants, Z will peak at the most likely recombination fraction frequency. Confidence intervals are accepted to extend the recombination fraction at which the lod score is 1 unit below the peak value (the lod-1 rule). The threshold of lod 3 for classical two-point linkage studies of simple mendelian traits corresponds to a pointwise significance level of $P = 10^{-4}$, for a genome wide significance level of about 9% (Chotai, 1984). Actually, it appears to be necessary to rise the lod score threshold to 3.3, corresponding to P = 5 x 10-5, to achieve the recommended genome-wide significance level of 5 % (Lander and Kruglyak, 1995). Standing this, Lander and Kruglyak in 1995 have proposed to adopt new standards of significance for lod score value and a new classification based on the number of times that one would expect to see a result at random in a genome-wide search.

There are four categories of linkage to take in to account, depending on the statistical occurrence of positive data in a whole genome screening.

A statistical evidence that would be expected to occur one time at random in a genome scan is defined as *suggestive linkage* .

A *significant linkage* is defined as the statistical evidence expected to occur 0.05 times (with a probability of 5%). For a *highly significant linkage,* it is required an evidence expected to occur 0.001 times in a genome scan. *Confirmed linkage* requires a combination of initial studies subsequently confirmed by additional samples with a P value of 0.01.

These new and more rigorous criteria for the linkage study will be worth to be adopted also to have in hand strong data before undertaking positional cloning to avoid a wasting of time and money to search for a phantom locus. Although obtaining a positive lod score is the ultimate goal in genetic mapping, exclusion data have to be favorably considered.

In fact, they tell us where the disease is not, and therefore narrow down the range of locations where it might be. If enough of the genome is excluded, only a few possible locations may remain.

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