Linkage disequilibrium between FD1-D9S202 haplotypes and the Friedreich's ataxia locus in a central-southern Italian population

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Abstract

We used two recently described genetic markers in the region of the Friedreich's ataxia locus to study 33 affected pedigrees from central-southern regions of Italy. These markers are predicted, by physical mapping, to be localised more closely to the Friedreich's ataxia locus than other previously described markers. No recombination was found between these markers and the disease locus. Strong linkage disequilibrium is present between the compound haplotype and the disease locus. Since this population was also previously studied by using three other more distal genetic markers, a total of five markers has been used to identify the extended haplotype. Homozygosity in consanguineous pedigrees was also studied. Extended haplotype analysis and homozygosity studies suggest the presence of few common disease causing mutations in our population.

(J Med Genet 1994;31:133-135)

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Received 19 March 1993
Revised version accepted for
publication 24 September
1993

Friedreich's ataxia (FRDA according to the GDB nomenclature) is a relatively rare autosomal recessive degenerative disorder which usually manifests before adolescence.¹ It is characterised by relentlessly progressive gait and limb ataxia, loss of position sense and of tendon reflexes in the lower limbs, dysarthria, kyphoscoliosis, pes cavus, hypertrophic cardiomyopathy, and increased risk of diabetes mellitus.

The birth incidence of FRDA in Italy as a whole has been estimated to be between 1/22 000 and 1/25 000.²

The FRDA locus has been assigned to 9q13-q21.1 by genetic linkage to the anonymous DNA marker MCT112 (D9S15).^{3,4} During the last four years, efforts to isolate the disease gene have included the cloning of several other genetic markers.⁵⁻⁷ Detailed physical maps of the region, in both genomic DNA and yeast artificial chromosome clones, have shown the chromosomal order of these markers to be GS4(D9S111)-80 kb-MCT112/MS(D9S15)-70 kb-GS2(D9S110)-160 kb-26P(D9S5).^{8,9}

By using a linkage disequilibrium approach we suggested that the FRDA locus is probably located at the 26P side of this cluster of markers¹⁰ and in a recent collaborative study we reported the evidence of genetic recombi-

nation events that position the FRDA locus out of the cluster, at the 26P side.¹¹ These findings concentrated the efforts to isolate markers in the genomic region proximal to that covered by the markers described at the 26P side. Two microsatellite markers, FD1 (X11) and MLS1 (D9S202), were thus identified.^{12,13} These markers are located respectively at 80 kb and 190 kb from 26P.

In the present study we used these two markers to study 132 subjects from 33 FRDA pedigrees from a restricted geographical area (central-southern Italy). The same population was also previously studied for GS4, MS, and GS2^{10,11} so we were able to analyse GS4-MS-GS2-FD1-MLS1 extended haplotypes. Linkage analysis showed no recombination between both FD1 and MLS1 and FRDA. The FD1-MLS1 haplotype was found to be in linkage disequilibrium with the FRDA locus.

Materials and methods

STUDY POPULATION

The material for this study came from 132 subjects from 33 FRDA pedigrees. The 42 FRDA patients were diagnosed according to the criteria of Filla *et al.*¹⁴ All subjects are white and live in a restricted geographical area covering three central-southern Italian regions (Lazio, Campania, and Calabria). All the parents were born in the same regions except for three who came from two northern regions of Italy (Piemonte and Veneto).

DNA ANALYSIS

A blood sample was taken in 50 mmol/l EDTA from all subjects. Genomic DNA was prepared according to previously described procedures.¹⁵ PCR was used to detect microsatellite polymorphisms. DNA from each subject was amplified in 10 µl of PCR buffer (10 mmol/l Tris-HCl, pH 8.3, 1.5 mmol/l MgCl₂, 50 mmol/l KCl, 0.001% gelatin) containing 10 pmol of ³²P labelled sense oligonucleotide primer, 10 pmol of antisense oligonucleotide primer, and 200 µmol/l of each deoxyribonucleotide primer, and 200 µmol/l of each deoxyribonucleotide triphosphate. One unit of *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) was added and 50 µl of mineral oil were layered over the samples. Samples were then incubated at 95°C at annealing temperature (57°C for MLS1 and 60°C for FD1) and at 72°C for 50 seconds; this

cycle was performed 36 times in a DNA-RNA amplifier (Biostar Violet, Rome, Italy).

The oligoprimers sequences are as follows (S = sense, A = antisense):

FD1: 5'-CAGGTTGTCTCAAGTTATCAACC-3'(S)
5'-GGTCTCTCAGGATGAGACTGAAC-3'(A)
MLS1: 5'-CTTGTCTGTGATCGTGGTCACCTGTACC-3'(S)
5'-AACATCTTGATAATTATAGAATAA-3'(A)

PCR product sizes (more frequent allele) were as follows: MLS1 = 201 bp; FD1 = 230 bp.

PCR products were loaded on 6% polyacrylamide-urea gels and analysed by autoradiography. Alternatively, PCR products were detected, after blotting on a nylon membrane and hybridisation with DIG labelled probes, by chemiluminescent and colorimetric methods (Pianese *et al*, manuscript in preparation).

STATISTICAL ANALYSIS

Pairwise lod scores were calculated, using the Mlink program from the LINKAGE package version 5.1,¹⁶ between FRDA and (1) the individual microsatellites and (2) the extended (GS4-MS-GS2-FD1-MLS1) haplotype. χ^2 analysis was performed to test linkage disequilibrium by using the SPSS statistical program.

Results

LINKAGE ANALYSIS

In the family set analysed we found no phase known obligate recombinant between FRDA and any of the markers and neither was any crossover between any marker pair found. The entire MLS1-FD1-GS2-MS-GS4 extended haplotype resulted in tight linkage with FRDA with $Z_{\max} = 9.3$ at $q = 0$.

LINKAGE DISEQUILIBRIUM

Linkage disequilibrium with the disease was first separately evaluated for FD1 and MLS1, but both markers gave non-significant results. We then examined MLS1-FD1 haplotypes. We were able to identify unambiguously 94 haplotypes (51 on FRDA and 43 on normal chromosomes). The table shows the observed MLS1-FD1 haplotype frequencies in normal and FRDA chromosomes. A statistically significant evidence of association ($\chi^2 = 20.75$, $p = 0.0009$) between FRDA and MLS1-FD1 haplotypes was found. In particular, we noted a higher frequency of haplotype 2-2 and 3-3 in FRDA chromosomes and an increase of haplotype 3-2 in normal chromosomes.

Linkage disequilibrium between MLS1-FD1 haplotype and FRDA

MLS1-FD1 haplotype	FRDA chromosomes	Normal chromosomes
2-1	2 (3.92)	3 (6.98)
2-2	19 (37.25)	3 (6.98)
2-3	4 (7.84)	9 (20.93)
3-1	3 (5.88)	0 (0)
3-2	10 (19.61)	20 (46.51)
3-3	13 (25.49)	8 (18.60)

$\chi^2 = 20.75$, $p = 0.0009$. Figures in parentheses are percentages.

EXTENDED HAPLOTYPE ANALYSIS

A total of 76 extended (MLS1-FD1-GS2-MS-GS4) haplotypes (42 on FRDA chromosomes and 34 on controls) were unambiguously assigned. Twenty-one different haplotypes were found on normal chromosomes and 26 on FRDA alleles. We were unable to show the presence of a "common" haplotype in our population. On the other hand, it is intriguing that a "core" haplotype 3-3-2-2-X (MLS1-FD1-GS2-MS-GS4) is present on nine (21.4%) FRDA chromosomes but never on normal chromosomes (Fisher exact test $p = 0.003$).

HOMOZYGOSITY IN PEDIGREES WITH CONSANGUINEOUS PARENTS

We identified eight pedigrees with consanguineous parents (R ranging from 1/8 to 1/128) and homozygosity was detected for MLS1 and FD1 in all families. In two pedigrees all markers apart from GS4 were homozygous. Heterozygosity was noted at both GS2 and GS4 in only one family ($R = 1/16$). Homozygosity of the entire extended haplotype was also detected in two families with apparently unrelated parents. In these cases we showed that all the grandparents were born in the same, very small, geographical area.

Discussion

The classical way to identify a "candidate" chromosomal region in which putative transcripts can be searched for is through the construction of a detailed genetic map. In particular it is very important to define the boundaries of this region in relation to recombination events. In the case of the FRDA locus this goal has not been achieved with the markers used so far, since even the most proximal marker, 26P, has been shown to recombine.¹¹ Linkage and linkage disequilibrium studies with recently isolated markers, which are, by physical mapping, closer to the FRDA locus, appear to be very important in defining at least one boundary of the FRDA locus.

Linkage analysis showed that both FD1 and MLS1 are significantly linked with the FRDA locus, with absence of any recombination event. This finding was expected, since in our set of pedigrees none of the other less proximal markers showed any recombination either. On the other hand, in several pedigrees in which there is recombination with the previously described markers, no recombination has been detected with MLS1 (M Pandolfo, personal communication).

Conflicting data have been obtained in the past about linkage disequilibrium between FRDA and marker loci which maybe because of the small size and the heterogeneity of the population samples. In a recent report, using a larger sample size, we were able to show strong disequilibrium between 26P and FRDA, and only a marginal association of the disease locus with the other markers, suggesting a positioning of the disease locus on the D9S5 side of the marker cluster. Such a conclusion is in agree-

ment with recombination data which clearly positions FRDA beyond D9S5.

In this study we found strong linkage disequilibrium between FRDA and the compound MLS1-FD1 haplotype in a relatively small population. Our finding can be explained by the relative genetic homogeneity of our population, which, if new mutations are rare, should share only a few FRDA causing mutations. In such a situation, a very close set of markers can show linkage disequilibrium with the disease. In turn, demonstration of linkage disequilibrium can be achieved with a relatively limited set of observations only if one or more mutations are associated with allele(s) at low frequency on normal chromosomes.¹⁷ This will be more likely when markers of high informativeness are used. Increased informativeness may be obtained by examining haplotypes instead of single polymorphisms as we have done with FD1 and MLS1. This way detected strong linkage disequilibrium between FRDA and the MLS1-FD1 haplotype that was not shown by the separate analysis of each marker, and which seems to indicate the proximity of these markers to the disease locus. This conclusion is in full agreement with the previous data¹⁰ and with the homozygosity analysis.

The principle of homozygosity studies is that where there is consanguinity, the disease gene and the surrounding region are inherited from a single chromosome of a common ancestor. Thus polymorphic sites in the region should be more frequently homozygous. As genetic markers are less closely linked to the disease locus, heterozygosity could appear by recombination events. In our consanguineous pedigrees only FD1 and MLS1 are always homozygous. In one family, heterozygosity starts at the GS2 site, including GS4, and a recombination event is the most likely explanation for this. In two other pedigrees, heterozygosity involves only the GS4 locus. Since, in these two cases, the two alleles detected differ from each other only by a repeat unit, a mutation event in the GS4 locus could be an alternative explanation. We underline that in a previous report we positively demonstrated a mutation event in GS4.¹¹ In any case homozygosity studies seem to indicate that GS4 tends to vary more than other markers. These data are consistent with those obtained by extended haplotype analysis. If we consider the entire MLS1-FD1-GS2-MS-GS4 haplotype, we find a large spreading of different haplotypes on FRDA chromosomes. If we exclude GS4 from the analysis, we could identify at least one haplotype (3 3 2 2 X) which is shared by 21.4% of FRDA chromosomes.

In conclusion, our results show that (1) the

two genetic markers MLS1 and FD1 are closely linked to the FRDA locus; (2) the compound MLS1-FD1 haplotype is in strong linkage disequilibrium with the disease locus; and (3) the more distal part of the marker cluster tends to vary in the population by recombination or mutation events. As concerns the last point, it could have an impact on extended haplotype analysis frustrating attempts to evaluate the genetic heterogeneity of FRDA.

This work was supported in part by CNR Italy (grant No 91.04180) and by a grant from Italian Telethon to MP. We thank F Duclos for making the FD1 marker available to us before publication. FC is a recipient of a fellowship from CNR.

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