Linkage analysis of a large pedigree with hereditary sideroblastic anaemia

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Abstract

A large pedigree showing a history of pyridoxine responsive X linked sideroblastic anaemia was screened with several polymorphic DNA markers from the X chromosome. Linkage analysis between each marker and disease status was performed, giving a maximum two point lod score of 3.64 at zero recombination with the microsatellite marker PGK1P1 at Xq11.2-12. Close linkage to PGK at Xq13.3, one of the candidate regions for X linked sideroblastic anaemia, was excluded. Linkage to DNA markers distal to PGK and at Xp21 was also excluded. Multipoint linkage analysis was performed with markers located between Xq11.2-21. The maximum map specific lod score obtained was 3.56 at PGK1P1 (Xq11.2-12). Linkage remained significant over the interval 20 cM proximal to PGK1P1 and 5 cM distal to PGK1P1, with definite exclusion around the PGK locus. The most likely location of the gene involved in sideroblastic anaemia in this pedigree is therefore within the pericentromeric region of the X chromosome. This region includes the erythroid 5-aminolaevulinate synthetase gene of the haem synthesis pathway, which is a candidate gene for X linked sideroblastic anaemia located at Xp11.21.

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The sideroblastic anaemias are either inherited or acquired. The inherited form generally follows an X linked recessive pattern although some cases of autosomal inheritance have been documented.¹ X linked hereditary sideroblastic anaemia is a rare disorder, manifesting mainly in males, usually in childhood or adolescence. Carrier females may be affected but usually show mild or no anaemia. The mode of inheritance in the pedigree investigated in this study is X linked recessive.²

The person affected with X linked hereditary sideroblastic anaemia often presents with a dimorphic, hypochromic, microcytic anaemia. The serum iron is usually raised, the total iron binding capacity is saturated, and the serum ferritin may also be slightly raised. In the bone marrow 10 to 40% of erythroblasts contain stainable iron granules arranged in a ring around the nucleus, the erythroblast mitochondrion being loaded with iron. Ringed sideroblasts are the diagnostic feature of the sideroblastic anaemias. Hereditary sideroblastic anaemia is not generally life threatening;

death from the condition is rare but can result from haemochromatosis owing to excess iron deposition in the tissues and organs.³

Microcytic hypochromic anaemia is generally associated with iron deficiency, but in hereditary sideroblastic anaemia iron deficiency is clearly not the cause; the patient is iron overloaded. The defect appears to be in iron utilisation, most probably in the synthesis of haem. Haem is synthesised in the mitochondria, so that a failure to utilise iron could cause the observed accumulation of iron in the mitochondria. The first enzyme of the erythroid haem synthesis pathway, 5-aminolaevulinate synthetase, is located on the X chromosome and is therefore a candidate gene for X linked sideroblastic anaemia. Astrin and Bishop⁴ isolated an aminolaevulinic acid synthetase, ALAS-2, that is only expressed in erythroid cells and assigned the gene involved to the X chromosome. The gene has since been mapped to Xp11.21^{5,6} and characteristics of the gene and protein have been described.⁷

A number of patients with X linked sideroblastic anaemia, including affected members of the pedigree investigated in this study, have shown some response to treatment with pyridoxine, vitamin B6. Pyridoxal phosphate is derived from pyridoxine and is a co-enzyme for ALAS-2. Cotter *et al*⁸ have described a sequence variant in exon 9 of the ALAS-2 gene in a single male with pyridoxine responsive X linked sideroblastic anaemia. Exon 9 is thought to code for part of the pyridoxal phosphate binding site. A single T to A base change in codon 471 resulting in an isoleucine to asparagine substitution was observed.⁸ Cox *et al*⁹ have since described a sequence variant in exon 8 of ALAS-2 in two affected males and one female carrier of a pedigree with pyridoxine responsive X linked sideroblastic anaemia. Exon 8 is also believed to code for part of the pyridoxal phosphate binding site. A single C to G base change in codon 388 was observed resulting in a threonine to serine substitution.⁹

However, hereditary sideroblastic anaemia appears to be genetically heterogeneous as linkage to other regions of the X chromosome has also been reported. Pagon *et al*¹⁰ described a pedigree in which hereditary sideroblastic anaemia and the non-progressive spinocerebellar syndrome, ataxia, were co-segregating in an X linked recessive fashion. Linkage to phosphoglycerate kinase at Xq13 (maximum lod score of 2.6 at a recombination fraction of zero) has since been reported in the pedigree.¹¹ The microsatellite in intron 7 of ALAS-2 was uninformative for linkage in the pedigree,¹² but linkage to DXS14, which maps

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Figure 1 Pedigree of the family with X linked sideroblastic anaemia.

close to ALAS-2, was excluded.¹¹ X chromosome abnormalities (including rearrangements involving Xq13) have been observed in some patients with idiopathic acquired sideroblastic anaemia.¹³ However, the basic biochemical defect involved in idiopathic acquired sideroblastic anaemia may be different from that in hereditary sideroblastic anaemia. Candidate regions for the gene responsible for hereditary sideroblastic anaemia in the pedigree investigated with linkage markers in this study were Xq13 and Xp11.21; other regions of the X chromosome were also investigated.

Materials and methods

PATIENTS

The part of the pedigree investigated is shown in fig 1; the proband is member III-1. A more complete pedigree may be seen in Losowsky and Hall.² Affected males were diagnosed on the basis of peripheral blood film appearance, the occurrence of ringed sideroblasts in the bone marrow, and serum iron levels. The pedigree members genotyped included six affected males, seven unaffected males, and six obligate carrier females.

MICROSATELLITE AND RFLP ANALYSIS

Whole blood was collected into EDTA and DNA extracted by standard methods. Details of the DNA markers used are listed in table 1.

PCR was carried out in a total reaction volume of 25 µl with 50 to 100 pmol of each primer, approximately 250 ng of DNA, *Taq* DNA polymerase reaction buffer (10 mmol/l tris-Cl pH 8.0, 45 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.01% triton-X 100) and 200 µmol/l of each dNTP (RFLPs) or 250 µmol/l dCTP, dGTP, and dTTP, 2.5 µmol/l dATP, 80 µmol/l (approximately 2.5 µCi, Amersham) of α-³²S dATP or α-³³P dATP (microsatellites) and 1.25 U of *Taq* DNA polymerase (Promega). PCR was carried out for 30 cycles (RFLPs) and 24 to 30 cycles (microsatellites). Primers were annealed at 50 to 60°C and extended at 72°C for RFLPs and annealed and extended at 55 to 60°C for microsatellites as described in Noble *et al.*²⁶ For RFLPs, the PCR product was digested with the appropriate restriction endonuclease and resolved through a 2% agarose gel. For microsatellites an equal volume of loading buffer (95% v/v deionised formamide, 0.1% w/v xylene cyanol, 0.1% w/v bromophenol blue, 100 mmol/l tris-borate (pH 8.3), 2 mmol/l EDTA) was added to the PCR product before denaturation and electrophoresis through an 8% denaturing polyacrylamide gel. The alleles were scored with allele 1 representing the largest allele within the pedigree and allele 2 representing the second largest allele, etc.

GENETIC LINKAGE ANALYSIS

Two point linkage analysis was performed with MLINK (LINKAGE version 5.03) with a model of recessive X linked inheritance. All marker alleles were assigned equal frequencies according to the number of alleles seen in the family for each marker (table 1). Recalculation with equal frequencies for larger numbers of alleles yielded slightly higher positive lod scores in this family. These were not used, as it is most likely that the allele associated with the disease will be a more common one and a more conservative approach was preferred. To facilitate linkage analysis the genotypes of the markers located at PGK (PY2 and PY5) were combined to produce haplotypes relating to a single marker, PGK combined. This is possible because, as expected, no recombination was observed between these two markers. Five alleles were assigned to this marker. The allele frequency of X linked hereditary sideroblastic

Table 1 DNA markers. All microsatellites are denoted by (CA)_n. The chromosomal location and relevant gene (where applicable) are shown. All primer sequences can be found in the references

Marker	No of alleles in the pedigree	Location/gene ¹⁴	Reference
Dys3' (CA) _n	2	Xp21.1 dystrophin	15
pERT87-15/ <i>Xmn</i> I	2	Xp21 dystrophin	16
Dys 5'II (CA) _n	3	Xp21.3 dystrophin	17
Dys 5'I (CA) _n	4	Xp21.3 dystrophin	17
MAOB (CA) _n	3	Xp11.4-11.3 monoamine oxidase B	18
DXS426 (CA) _n	2	Xp11.23-11.3	19
ALAS-2 (CA) _n	3	Xp11.21 5-aminolaevulinatase synthetase	12
PGK1P1 (CA) _n	2	Xq11.2-12	20
DXS453 (CA) _n	4	Xq11.2-12	21, 22
PGK-PY2 (CA) _n		Xq13.3 phosphoglycerate kinase 1	23
PGK-PY5 (CA) _n		Xq13.3 phosphoglycerate kinase 1	23
DXS456 (CA) _n	3	Xq21-22	19
DXS424 (CA) _n	4	Xq24-26	19
FIX/ <i>Hha</i> I	2	Xq27 factor IX	24
FVIII/ <i>Bcl</i> II	2	Xq28 factor VIII	25

Figure 2 Key pedigree members with the haplotypes for the markers PGK1P1, DXS453, and PGK combined (PGK-PY2 and PGK-PY5) shown below each member.

anaemia was taken to be 1/10 000 with a mutation rate of 1×10^{-6} . Complete penetrance was assumed for males; the penetrance in heterozygotes was given as zero, as no attempt was made to ascertain carrier status from clinical data.

For multipoint linkage analysis, the same parameter values were used and lod scores were calculated with the sideroblastic anaemia locus moved between the loci PGK1P1, DXS453,

PGK combined, and DXS456 using LINK-MAP (version 5.10). The recombination fractions between PGK1P1-DXS453, DXS453-PGK, and PGK-DXS456 were estimated from published data. The distance between PGK1P1 and PGK was estimated to be 7 cM, and PGK1P1 is thought to be proximal to DXS453, although this is not finally confirmed.^{14 22 27} For the purpose of multipoint linkage analysis, PGK1P1 and DXS453 were placed 5 cM apart and PGK was placed 2 cM from DXS453. However, moving DXS453 within the 7 cM interval assumed between PGK1P1 and PGK did not significantly affect the results. DXS456 was placed 10 cM from PGK.

Results

Genotypes were obtained from the descendants of II-1 and II-3 only for the markers Dys3', pERT87-15/XmnI, Dys 5'II, Dys 5'I, DXS426, DXS456, DXS424, FIX/HhaI, and FVIII/BclI. Genotypes of the remaining markers were obtained for the whole pedigree. The haplotypes, of key members only, for PGK1P1, DXS453, and PGK combined are shown in fig 2.

The results of two point linkage analysis between X linked sideroblastic anaemia and each marker over a range of values for θ from 0 to 0.3 are shown in table 2. The results of multipoint linkage analysis are shown in fig 3.

Table 2 Two point analysis of hereditary sideroblastic anaemia versus marker

Marker	0.000 θ	0.001 θ	0.05 θ	0.10 θ	0.20 θ	0.30 θ
Dys 3' and DXS426	0.13	0.13	0.10	0.07	0.02	-0.01
pERT87-15/XmnI	- ∞	-3.87	-0.60	-0.14	0.15	0.16
Dys 5'II	0.70	0.70	0.63	0.55	0.41	0.27
Dys 5'I	- ∞	-1.60	-0.00	0.19	0.25	0.19
DXS456	- ∞	-1.25	0.35	0.54	0.59	0.48
DXS424	- ∞	-5.36	-0.47	0.21	0.64	0.64
FIX/HhaI	- ∞	-6.74	-1.72	-0.91	-0.24	0.01
FVIII/BclI	- ∞	-2.59	-0.91	-0.63	-0.36	-0.21
MAOB	0.89	0.88	0.78	0.68	0.49	0.31
ALAS-2	0.76	0.76	0.67	0.58	0.41	0.25
PGK1P1	3.64	3.63	3.33	3.01	2.34	1.63
DXS453	3.51	3.50	3.16	2.81	2.07	1.28
PGK combined	- ∞	-0.94	2.10	2.32	2.12	1.6

Figure 3 Plot of multipoint analysis. Lod score is plotted against θ from PGK1P1. Distances from PGK1P1 are 0.050 for DXS453, 0.070 for PGK, and 0.170 for DXS456.

Discussion

Pedigree member I-2 was an obligate carrier of HSA as she had an affected son. It is possible to infer unambiguously the haplotypes in I-1 and I-2 for PGK combined (fig 2). The two obligate carrier daughters, II-1 and II-3, have inherited different maternal alleles at the PGK combined locus. The alleles were also different for the DXS456 and DXS424 loci (not shown). There has clearly been a recombination event between the disease locus and these loci in at least one of the daughters. For the PGK1P1 and DXS453 loci, only one of the haplotypes in I-2 can be inferred, because it is inherited by all the branches of the family with affected members that have been analysed. It is also present in all the affected males, and absent from the unaffected males. This suggests that this chromosomal region could be implicated in the aetiology of the disease.

Two point linkage analysis of hereditary sideroblastic anaemia versus marker was performed (table 2). The microsatellites at PGK1P1, DXS453, and markers at PGK provided information around Xq13. The maximum two point lod scores were 3.64 at zero recombination with microsatellite PGK1P1 (located at Xq11.2-12) and 3.51 at zero recombination with microsatellite DXS453, assigned to the same region. The slight difference in the lod scores is because of differences in assumed allele frequencies as both markers were fully informative. Linkage was excluded with the markers located at PGK (Xq13.3) at 0% recombination. Linkage to markers distal to Xq13 (DXS456, DXS424, FIX/HhaI, and

FVIII/*BclI*) and to the dystrophin (Xp21) markers pERT87-15/*XmnI* and the Dys 5'1 microsatellite was also excluded. The microsatellites Dys 3', Dys 5'II, DXS426, MAOB, and ALAS-2 produced no significant information; in particular, all affected members and obligate carriers possessed an allele of the ALAS-2 microsatellite in common, but the marker had a low degree of heterozygosity in the family. Map specific multipoint linkage analysis between hereditary sideroblastic anaemia, PGK1P1, DXS453, PGK combined, and DXS456 was also performed (fig 3). The maximum lod score obtained from multipoint analysis was 3.56 at PGK1P1. This value is strongly supportive of linkage in the PGK1P1 region. The 10-fold support interval (lod >2.6) extends 20 cM proximal to PGK1P1, and 5 cM distal to PGK1P1 with definite exclusion of the PGK locus. The lod scores suggest that the disease locus in this family is 40 times more likely to be located proximal rather than distal to PGK.

These results very strongly suggest that the candidate region for hereditary sideroblastic anaemia in this pedigree is the pericentromeric region of the X chromosome. This region includes the ALAS-2 gene localised to Xp11.21. The reports of linkage to PGK at Xq13¹¹ and of sequence variants in ALAS-2 at Xp11.21^{8,9} indicate HSA is likely to be a genetically heterogeneous disorder. Linkage to PGK (Xq13) has been excluded in the pedigree investigated in this study, so that ALAS-2 is a strong candidate gene for the X linked sideroblastic anaemia found in this pedigree.

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