Fabry disease in a large Nova Scotia kindred: carrier detection using leucocyte α -galactosidase activity and an *NcoI* polymorphism detected by an α -galactosidase cDNA clone

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Abstract

Fabry disease is an X linked recessive disorder of glycosphingolipid metabolism resulting from a deficiency of the lysosomal hydrolase α -galactosidase (α -gal). Measurement of the enzyme activity, however, is not an accurate method for identification of female carriers among at risk relatives of affected males. The α -gal cDNA and gene have been cloned previously and found to provide useful probes for the molecular analysis of affected families but these clones have not been available to us. Thus, to analyse Fabry disease in Nova Scotia, especially within a large kindred known to contain 30 affected males and 50 possible carrier females, we isolated an independent cDNA for α -gal. Using this clone as a probe, the mutation in the Nova Scotia kindred was shown not to be a major DNA alteration, but was found to be linked to the rarer allele (frequency 0.20) of the polymorphic *NcoI* site located 3' to the gene. Affected males from two Nova Scotia families

who cannot be associated with the kindred by history were also found to have the rarer *NcoI* allele, which suggests they are, in fact, part of the kindred. The coupling of the mutation to an infrequent marker also helped carrier identification in the kindred where all of 17 obligate carriers examined, including six who were not identified as carriers by enzyme assays, were found to be heterozygous for the RFLP. Thus, DNA analysis can be used for presymptomatic and prenatal diagnosis in most portions of the Nova Scotia kindred affected with Fabry disease.

Fabry disease (Anderson-Fabry disease; angiokeratoma corporis diffusum universale) is an X linked recessive disorder of glycosphingolipid metabolism in which the deficiency of a lysosomal hydrolase, α -galactosidase (α -gal), leads to the progressive accumulation of ceramide trihexoside (CTH; globotriaosylceramide) in tissues and fluids of affected subjects,¹ with subsequent deposition in blood vessels, neurones, cornea, kidney and myocardium.² The α -gal locus has been mapped to Xq21.33.³⁻⁵ Hemizygous males usually suffer from painful crises in the extremities, develop the characteristic angiokeratoma of the condition with progressive renal and cardiac disease secondary to glycolipid accumulation, and rarely survive beyond the fifth decade of life. Heterozygous females exhibit fewer signs and symptoms of the disease, although occasional females have been described with symptoms similar to the male.¹

The reliable ascertainment of this disease by enzyme analysis in heterozygotes is difficult, probably because of random inactivation of the X chromosome⁶ or X chromosome reactivation,⁷ or both. Clinical symptoms and signs may be non-existent¹ and α -gal levels can be indistinguishable from normal in body fluids and tissues.⁸ The reliability of detection may be improved by measurement of activity in hair roots,^{8,9} cloning of cultured fibroblasts,¹⁰ or cell sorting.¹¹ However, these procedures are tedious, time consuming, and technically demanding. DNA analysis

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can provide more accurate heterozygote identification through the use of restriction fragment length polymorphisms (RFLPs) detected either at the α -gal locus¹ or by closely linked anonymous DNA sequences.^{4, 12}

A partial¹³ and full length cDNA^{14, 15} encoding α -galactosidase have been isolated from human liver λ gt11 expression and SV40 transformed human fibroblast pcD cDNA libraries, respectively. Northern hybridisation studies¹⁶ have shown that a 1.4 kb α -gal mRNA transcript is produced in normal cells. A normal size and level of α -gal mRNA production was found in 13 of 30 unrelated Fabry hemizygotes examined. The remainder had either no detectable transcript, or decreased levels of a normal sized mRNA, or decreased/normal levels of an aberrant sized mRNA. Southern hybridisation analyses of DNA from 130 unrelated Fabry hemizygotes have detected seven gene rearrangements,¹⁷⁻²⁰ including five deletions, one duplication, and one point mutation as shown by an aberrant *MspI* restriction site.

Two RFLPs have been identified with the full length α -gal cDNA. *SacI* and *NcoI*^{19, 20} detect polymorphisms within and 3' to the α -gal gene, respectively. The *SacI* RFLP was found in 8% of normal alleles and in 2% of unrelated Fabry families examined. The *NcoI* RFLP was found in 13% of normal alleles and in 12% of Fabry families examined. No linkage disequilibrium and no recombinants between the RFLP and disease locus were detected. RFLP analysis of Fabry families may aid in carrier detection in informative families who show no gross rearrangement of the α -gal gene.

We have previously described²¹ a large Nova Scotia kindred in which there are approximately 30 living affected males and over 50 possible carrier females. Molecular analysis of the α -gal alleles transmitted in this kindred would enhance the accuracy of heterozygote identification, allow for the characterisation of molecular lesions in this large group, and aid in the genetic epidemiology of the historically unrelated cases of Fabry's disease in the Canadian Maritime provinces. Although we were unable to obtain cloned α -gal sequences described previously, we report the independent isolation of a cDNA for α -gal and its use for the DNA analysis of Fabry disease in Nova Scotia.

Materials and methods

ISOLATION OF AN α -GALACTOSIDASE cDNA

A human cDNA library constructed by Okayama and Berg²² from SV40 transformed human fibroblasts was kindly provided by Dr R Gravel (Research Institute, Hospital for Sick Children, Toronto, Canada M5G 1X8). The Okayama and Berg cDNA library contains approximately 1.4×10^6 independent clones. A frozen aliquot of this cDNA library was 'scraped' with the tip of a Pasteur pipette and the scrape added to

liquid medium. The medium was placed in a shaker incubator overnight at 37°C. Aliquots were removed at hourly intervals for six hours and from the overnight culture. These aliquots were plated, a time-growth curve constructed, and the number of cells present in the original 'scrape' determined. When plating the cDNA library, an appropriate number of 'scrapes' were taken to ensure that at least 10^6 cells were present at time 0 hours. This was done in order to optimise conditions for finding the α -gal cDNA from the 1.4×10^6 independent clones available. The library was plated at a cell density of 10^4 colonies/100 mm² plate from an overnight liquid culture of the library 'scrapes'. Sterile nitrocellulose filters were overlaid on each 100 mm² plate and the plates incubated at 37°C overnight. The nitrocellulose filters were then transferred, colony side up, to plates containing spectinomycin (100 μ g/ml) for plasmid amplification and further incubated at 37°C overnight. The master plates were sealed with parafilm and stored at 4°C. The amplification plates were discarded. The colony lifts were denatured in 0.5 mol/l NaOH/1.5 mol/l NaCl, vacuum dried at 80°C for two hours, and stored at room temperature.

A 30mer oligodeoxynucleotide (5' AGGACTGG-CAGAAGCATTGTGTACTCTCTGT 3') was commercially synthesised (Regional DNA Synthesis Laboratory, Department of Medical Biochemistry, University of Calgary, 3330 Hospital Dr NW, Calgary, Alberta, Canada T2N 4N1) using the nucleotide sequence from a partial cDNA clone for α -gal isolated by Calhoun *et al.*¹³ The 30mer oligonucleotide was 5' end labelled by T4 polynucleotide kinase using [α -³²P]ATP (New England Nuclear, 3000 Ci/mmol) to a specific activity of 4 to 5×10^8 cpm/ μ g. The colony lifts (20 filters/100 ml buffer) were prehybridised at 61°C (the calculated T_M of the 30mer oligonucleotide) for four hours in $6 \times$ SSC (0.9 mol/l NaCl, 0.09 mol/l Na citrate pH 7.0)/10 $\times$ Denhardt's (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, fraction V)/50 mmol/l phosphate buffered saline, pH 7.0. The radiolabelled probe was hybridised (10^6 cpm/ml buffer) overnight in fresh buffer at 61°C, washed in two changes of $6 \times$ SSC at 61°C, and exposed on Kodak XAR-5 film with an intensifier screen at -70°C for one to three days.

Colonies which gave a positive signal were 'picked' with the wide bore of a Pasteur pipette, amplified as overnight cultures, and replated at a cell density of 10^3 /100 mm² and subsequently 10^2 /100 mm² plate. Single colony isolates which gave a positive signal after these three rounds of purification were amplified and the plasmid DNA isolated according to standard procedures.²³ The plasmid DNA was restricted with *Bam*HI to release the insert DNA and electrophoresed in a 0.8% agarose gel. The radiolabelled 30mer was hybridised in situ to the gel to confirm the identity of the α -galactosidase cDNA insert.

The insert DNA was characterised further by single and double digestions with a number of restriction enzymes including *Nco*I, *Sac*I, *Bgl*II, *Bgl*III, *Pvu*II, *Msp*I, *Eco*RI, *Hind*III, and *Kpn*I. All restrictions were performed according to the manufacturer's recommendations (Boehringer, Mannheim).

PREPARATION OF HIGH MOLECULAR WEIGHT DNA

High molecular weight DNA was prepared from human leucocytes. Peripheral venous blood was collected in EDTA treated vacutainer tubes. The red

blood cells were pelleted by centrifugation and the white cell 'buffy coat' removed to a fresh tube. An isotonic solution of 0.04% NH_4Cl /10 mmol/l Tris, pH 7.6 (9:1) was added to the white cell suspension and the cells were incubated at 37°C for 15 minutes. The cell suspension was centrifuged, the haemolysed supernatant removed, and the white cell pellet resuspended in proteinase K buffer (10 mmol/l Tris, pH 8.0/100 mmol/l NaCl/1 mmol/l EDTA, pH 7.6) containing 0.5% sodium dodecyl sulphate (SDS) and 0.5 mg/ml proteinase K and incubated at 37°C overnight. The cell lysate was extracted with an equal

Figure 1 Partial pedigree of the Nova Scotia kindred affected with Fabry disease showing the relationship between the six family groups studied in the present report. ■=affected male; ○=carrier female; ?=diagnosis uncertain; △=sex unknown.

Figure 2 Pedigree of two additional family groups from Nova Scotia who are also affected with Fabry disease. Subjects II.7 and III.23 were adopted and there is no information regarding their natural biological parents. ■ = affected male; ○ = carrier female; ∫ = adopted.

*Nco*I RFLP with a heterozygote frequency of 0.24 was also reported by Bernstein *et al.*¹⁷ The remaining two clones (pAJK-1 and pAJK-2) contained inserts which were less than full length.

ASSOCIATION OF THE NOVA SCOTIA FABRY DISEASE LOCUS WITH THE *Nco*I RFLP

The α -galactosidase cDNA probe was first tested as a tool for carrier detection using leucocyte DNA from

members of six families from the large Nova Scotia kindred (fig 1). At the same time, leucocyte DNA from two other families was examined (fig 2). Patient II.7 was adopted and no information is available concerning her natural biological parents. Patient III.23 was from a broken home and vigorously maintains her isolation from her biological relatives. These eight family groups included 15 affected hemizygous males and 19 obligate heterozygote

Disease status based on history, enzyme assay, and RFLP inheritance.

Family group	Patient (see figs 1 and 2)	Sex	Status by history	α/β gal ratio	Status by enzyme assay	Genotype	Status by RFLP
1	III.2	F	O	0.21	N	B1B2	C
	IV.1	F	N	NT	?	B1B1	N
	IV.14	F	N	0.09	C	B2B2	C
	V.1	F	N	0.19	C	B1B2	?
2	III.4	F	O	0.05	C	B1B2	C
	IV.16	M	N	0.23	N	B1	N
	IV.17	F	N	0.52	N	B1B2	?
	IV.19	M	A	0.02	A	B2	A
	IV.20	F	N	0.29	N	B1B1	N
	IV.21	M	A	0.01	A	B2	A
	V.2	F	N	0.08	C	B1B2	C
	III.7	F	N	0.28	N	B1B2	?
	III.9	F	O	0.22	N	B1B2	C
	IV.24	F	N	NT	?	B1B1	N
3	IV.25	M	A	0.01	A	B2	A
	V.3	F	O	0.06	C	B1B2	C
	V.4	M	N	NT	?	NT	?
	V.5	F	O	0.23	N	B1B2	C
	V.6	M	N	0.43	N	NT	?
	IV.26	F	O	0.2	N	B1B2	C
	V.7	F	N	0.14	C	B1B2	?
	V.8	F	N	0.12	C	B1B2	?
	V.9	M	A	0.01	A	B2	A
	V.10	M	A	0.01	A	B2	A
	IV.28	F	N	0.1	C	NT	?
	IV.29	M	A	0.01	A	B2	A
	IV.30	F	N	0.23	N	B1B2	N
	V.12	F	O	0.15	C	B1B2	C
	V.13	F	O	0.04	C	B1B2	C
	V.14	F	O	0.08	C	NT	?
	III.13	M	A	0.01	A	B2	A
4	III.12	F	N	0.25	N	B1B1	N
	IV.31	F	O	0.01	C	B1B2	C
	IV.32	F	O	0.23	N	B1B2	C
	IV.33	F	O	0.12	C	B1B2	C
	V.15	M	A	0.01	A	B2	A
	V.16	F	N	0.11	C	B1B2	?
5	III.15	F	O	0.05	C	NT	?
	IV.34	F	N	0.11	C	B1B2	?
	IV.35	M	A	0.02	A	NT	?
6	V.16	F	N	0.19	C	B1B1	N
	III.16	F	O	0.2	C	NT	?
	IV.36	F	N	0.43	N	B1B1	N
	IV.37	M	A	0.01	A	B2	A
7	V.17	F	O	0.16	C	B1B2	C
	V.18	F	O	0.12	C	B1B2	C
	III.22	M	N	0.32	N	B1	N
	III.23	F	O	0.13	C	B1B2	C
	IV.43	M	A	0.004	A	NT	?
	IV.45	F	O	0.05	C	B1B2	C
	IV.46	M	N	0.33	N	B1	N
	IV.47	M	A	0.04	A	NT	?
	IV.48	M	A	0.01	A	B2	A
	V.20	F	N	0.09	C	NT	?
8	V.21	M	A	0.01	A	B2	A
	V.22	F	N	0.33	N	B1B1	N
	II.7	F	O	0.41	N	B1B2	C
	III.18	M	A	0.01	A	B2	A
	III.21	M	A	0.005	A	B2	A
	IV.39	F	O	0.11	C	B1B2	C
	V.19	F	N	0.15	C	B1B2	?

F=female, M=male, +=normal α/β ratio ≥ 0.208 , ?=unknown carrier status. N=normal, A=affected, O=obligate carrier, C=carrier.

Figure 3 Southern hybridisation analysis of *SacI*, *PvuII*, and *HindIII* digested DNA from affected hemizygotes IV.29 (fig 1) and III.21 (fig 2) and a normal control male. The affected males show a normal α -gal gene profile.

females whose carrier status had been previously determined by history and pedigree information (table). Obligate heterozygotes were defined as: (1) mothers of two or more affected sons; (2) mothers of one affected son and sisters of aunts of affected males; or (3) daughters of proven affected males. In addition, all of the hemizygous affected males had α -gal levels ≤ 2 nmol/mg protein/h and an α -gal/ β -gal ratio ≤ 0.02 (the cut off value in normal subjects is >0.2).⁸ α -gal levels and α -gal/ β -gal ratios were also very low in the affected males in these two families.

Affected males IV.29 (fig 1) and III.21 (fig 2) do not show any alterations of the *SacI*, *PvuII*, *HindIII* (fig 3), or *MspI* (data not shown) restriction fragment lengths as determined previously by Desnick *et al.*²⁰ The mutation present in these two families is not detectable by Southern analysis and therefore does not represent a major rearrangement of the α -gal gene.

The inheritance of the B1 and B2 alleles in one informative family from the Nova Scotia kindred is shown in fig 4. Patients III.2 and III.4 are obligate

Fabry carriers (table) and both are heterozygous for the B1 and B2 alleles. The two affected sons of III.4, patients IV.19 and IV.21, are both hemizygous for the rarer B2 allele. The clinically unaffected son (also unaffected by enzyme assay), IV.16, is hemizygous for the more common B1. The daughter of III.4, patient IV.17, is heterozygous for the B1 and B2 alleles and is at risk for being a Fabry carrier. Her father, III.5, is dead. Patient V.2, the daughter of affected male IV.21, and normal female IV.20, is heterozygous for the B1 and B2 alleles and a Fabry carrier, having inherited the B1 allele from her mother and the B2 allele, and hence the Fabry disease locus, from her affected father.

In addition, affected males from two Nova Scotia families (fig 2) which cannot be linked to the main kindred by history were also found to carry the B2 allele (table). This suggests that these two additional families are affected by the same mutant allele transmitted in the main kindred.

A direct estimate of the recombination rate was obtained from family groups 1 to 6 (fig 1) using the

Figure 4 Southern hybridisation analysis showing inheritance of the mutant Fabry gene with the B2 (23 kb) *NcoI* allele in affected hemizygotes IV.19 and IV.21, and inheritance of the normal Fabry gene with the B1 (13 kb) *NcoI* allele in unaffected hemizygote IV.16. Carrier status for Fabry disease was shown for III.4, III.2, IV.14, and V.2 and excluded for IV.1 because she was homozygous for the B1 (13 kb) allele. Subjects IV.1 and IV.14 are sisters and are homozygous for opposite alleles, B1 and B2 respectively. Homozygosity for the B2 allele in IV.14 precludes the determination of carrier status in V.1 without haplotype analysis for the X chromosome carrying the mutant Fabry gene. Carrier status for IV.17 also cannot be determined accurately as her father is dead.

MLINK sex linkage program.^{26 27} It should be noted that offspring of affected males would not be used in our lod score calculations as the Fabry disease locus lies outside the pseudoautosomal region and affected hemizygous males would therefore be uninformative in the determination of a recombination fraction between the disease locus and the *NcoI* polymorphism. Owing to the uncertainty observed in establishing clinical status in heterozygotes based on enzyme assay alone, only obligate carrier females were scored as informative. The maximum lod score obtained in our study is 3.66 at $\theta=0.00$ with a probability of linkage of 4571:1.

Discussion

A 1350 bp α -galactosidase cDNA was isolated from the Okayama and Berg²² SV40 transformed human

fibroblast cDNA library. Restriction analysis of this cDNA, designated pAJK-3, showed that it was identical to the full length α -gal cDNA reported by Tsuji *et al.*¹⁴ The full length cDNA contains 60 bp of a 5' untranslated region, a 93 bp sequence for a signal peptide, and a 1287 bp coding region for an unglycosylated precursor and mature enzyme subunit of $M_r=48\,772$ and 45 356 respectively.¹⁴⁻¹⁶ The genomic α -gal gene is approximately 12 kb and contains seven exons.¹⁶ Exon 1 contains the 5' untranslated region, the signal peptide sequence, and the first 33 residues of the mature enzyme. Intron 2 was found to contain three Alu repetitive sequences. The 5' flanking region contains regulatory elements, including a TAATAA sequence, five CCAAT box sequences, an Sp1 binding sequence, and several potential enhancer binding sites. An HTF (*HpaII* tiny fragment) island (unmethylated CpG dinucleo-

tide sequences which are located upstream in 'house-keeping' genes) was found at bp -660 in the α -gal gene. Using pAJK-3 as a hybridisation probe, we have confirmed the presence of a polymorphic *NcoI* restriction site near the 3' end of the α -gal gene^{19, 20} located in the region Xq21.33.³⁻⁵ Homozygosity for the B1 (IV.1, IV.20, IV.24, IV.36, V.16, and V.22) and B2 (IV.12 and V.14) alleles establishes these two *NcoI* fragments as alleles with frequencies of 0.80 and 0.20, respectively, and a PIC value of 0.27.

The B2 *NcoI* allele was found to segregate with the Fabry disease locus in the large Nova Scotia kindred reported by Spence *et al.*²¹ All of the subjects classified as carrier females or affected males by history and pedigree information had the B2, or 23 kb, *NcoI* allele (table). Several obligate carrier females (III.2, V.5, III.9, IV.26, IV.32, and II.7), who had apparently normal enzyme levels, were also shown to carry the B2 allele (table). There was no evidence for recombination between the B2 allele and the Fabry disease locus in any clinically established Fabry patient.

Using an α -gal/ β -gal ratio of 0.20 as the cut off between carrier females and controls,⁸ five of 21 obligate carriers had ratios in the control range and a further two fell on the dividing line. Thus 7/21 or 1/3 (33.3%) of the obligate heterozygotes in this group would have been misclassified or uncertain on the basis of enzyme analysis alone. This figure is substantially larger than the 15% we reported previously.²¹ The difference is not because of a change in the method or accuracy of measuring enzyme activity as similar methods were used. Some of the subjects reported here had also been studied earlier²¹ and their enzyme values have remained essentially unchanged. The higher figure probably reflects a selection bias during this study, since the subjects from whom blood was obtained were the most readily accessible and there was a greater proportion of persons with a high α -gal/ β -gal ratio among them. At this time, we feel that the 15% figure is still a reasonable one. The higher concordance between the clinical evidence of heterozygosity and the DNA data as compared to the enzyme data clearly illustrates the utility of the RFLP approach. It is interesting that the mean age of the obligate heterozygotes who tested indistinguishably from the controls in the enzyme assays was 40 years, whereas in the rest of the group it was 24 years. This age related difference may be important, since age related changes in the stability of X chromosome inactivation have been reported.⁷

Accurate heterozygote detection and prenatal diagnosis are now possible in this kindred using the *NcoI* RFLP. Recombination between the mutation and the *NcoI* polymorphism appears to be negligible and linkage of the mutation to the rarer allele suggests that the majority of carrier females should be heterozygous for the polymorphism (17/17 obligate carriers

analysed molecularly were B1B2) and thus allow predictive diagnosis by linkage analysis. This RFLP has even more general utility as it has also been identified by us in several isolated cases of Fabry disease in Nova Scotia with no known relationship to our kindred.

Based on the association between the B2 *NcoI* allele and the Fabry disease locus, carrier status was excluded for subject V.16. This patient had borderline levels of enzyme activity (0.19 as compared to a control value of 0.20, table) but was shown to be homozygous for the B1 allele, thereby eliminating her risk of being a carrier. Subject V.1 also had borderline levels of enzyme activity and was heterozygous for the B1 and B2 alleles. However, carrier status could not be established in this patient, as her mother (IV.14) was homozygous for the B2 allele and the origin of the patient's B2 allele could not be determined. Determination of carrier status in V.1 may be aided by establishing a haplotype of the affected X chromosome from one of her affected uncles (IV.4, 6, 9, 12). Haplotype analysis would also be helpful in delineating the carrier status of IV.17, whose father is dead.

Patient IV.1, the daughter of obligate carrier III.2 (B1/B2), is homozygous for the B1 allele and therefore not a Fabry carrier. Patient IV.14, also the daughter of obligate carrier III.2 (B1/B2), is a Fabry carrier by enzyme assay (table) and is homozygous for the B2 allele.

Restriction analysis of our Fabry patients' DNA showed no gross deletions or rearrangements. A previous study of 130 unrelated families with Fabry disease has shown that only seven (5.39%) have detectable molecular rearrangements by Southern analysis.¹⁸ Five partial gene deletions, ranging in size from 0.4 to 5.5 kb, were identified. Four of these deletions had a breakpoint in intron 2, suggesting that the Alu sequences found in this intron may lead to unequal crossover events. The fifth deletion had a breakpoint in exon 5. A gene duplication of exons 2 to 6 was also identified. A point mutation resulting in the loss of an *MspI* restriction site in exon 7 was found in a Fabry hemizygote who had approximately 2% residual α -gal activity. The altered *MspI* site was a missense mutation resulting from a C→T transition at position 1066 in the cDNA with a consequent arginine to tryptophan substitution at residue 356. No α -gal mRNA was detected in any of the six Fabry hemizygotes carrying these gene rearrangements. RNase A protection studies showed a deletion of the 198 bp exon 6 in the seventh Fabry family with a normal gene profile on Southern analysis but a shortened mRNA transcript. These reports indicate that Fabry disease is caused by a heterogeneous set of mutations in the α -gal gene, but that the majority of mutations are as yet unidentified. Lemansky *et al.*²⁸ have also shown by immunological detection of α -gal and its biosynthetic intermediates in five unrelated

Fabry males that Fabry disease results from a heterogeneous group of mutations which affect the synthesis, processing, and stability of α -galactosidase.

Further mRNA studies are currently under way to identify the molecular lesion of the α -galactosidase present in our Nova Scotia kindred. This will allow for more precise carrier analysis in at risk females who are not informative for the *NcoI* allele.

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