Original articles

Close linkage of a gene for X linked deafness to three microsatellite repeats at Xq21 in radiologically normal and abnormal families

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Abstract

We have used three highly polymorphic microsatellite repeats from Xq21 to type families in whom a gene for X linked deafness with perilymphatic gusher (DFN 3) was segregating. All three markers were tightly linked to the disease in its radiologically normal and abnormal forms, with a maximum lod score of 10.37 with DXS995 and 8.44 with DXS986 at zero recombination, and 14.03 with DXS1002 at $\theta=0.01$. In an isolated case of deafness of this type, DXS995 indicated either the first recombination observed between the marker and the disease gene or a new mutation in the proband. Southern blotting using a cosmid fragment from the candidate region has confirmed a de novo mutation by showing a deletion in the proband which is not present in his mother as judged by dosage analysis. We also describe a family with a paracentric inversion associated with a microdeletion and discuss how deletion mapping using these and other markers in the region has helped to define a candidate region for the gene.

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In clinical practice, deafness can usefully be classified into non-syndromic (in which there are no extra-auditory features) and syndromic, of which there are over 400 entries in the London Dysmorphology Database.¹ X linked deafness accounts for a small proportion of all non-syndromic genetic deafness, but it is nevertheless one of the best defined in terms of genetic and physical localisation. X linked deafness was initially classified into four groups on the basis of the pure tone audiogram,² but recent work has questioned the basis of that classification and proposes a radiological classification instead.³ Phelps *et al*⁴ described a unique abnormality on the CT scan of the petrous temporal bone in more than 50% of families with X linked deafness which consisted of dilatation of the internal auditory meati and deficiency of the bone between the basal turn

of the cochlea and the internal auditory meatus. This abnormality has been shown to be consistent for affected males within each pedigree so that it was either present in all deaf males scanned or in none. The bony defect may explain the well described phenomenon of the perilymphatic "gusher" at stapes surgery since it proposes a communication between the cerebrospinal fluid and the perilymphatic space. Whether there are two genes in Xq21 causing deafness accounting for the two radiological types, normal and abnormal, or whether there is a single gene in which allelic mutations may cause different pathology, is a question that will only be satisfactorily answered once the gene(s) have been cloned. What is clear from previous linkage studies is that deaf males with the typical bony abnormality on CT scan have a mutation in a gene at Xq21 in what is designated the DFN 3 locus. Other non-syndromic forms of X linked deafness without distinctive bony changes show linkage heterogeneity, with only some mapping to Xq21. Of the two reported families that do not map to Xq21,^{5,6} one family maps to Xp11.3-21.1⁶ and the other family maps to neither of these loci (unpublished data). Only families in whom the bony abnormality has been shown or families which have previously shown independent linkage to markers from Xq13-21 have been examined in this study.

Localisation of a major non-syndromic X linked deafness locus to the broad area Xq13-q21 is supported by linkage data from several groups,^{5,7-9} by analysis of cytogenetically visible male viable deletions with complex phenotypes, and the detection of microdeletions in unrelated male patients with X linked deafness.¹⁰ We now describe the use of three highly informative microsatellite repeats¹¹ to type seven families with this condition and show tight linkage to the gene. One of these markers, DXS995, was used to type an additional small kindred in which a deaf male showed the radiological abnormality characteristic of X linked deafness. The result indicated either the first recombination seen with this marker and the disease gene or a de novo mutation in the proband. Southern blotting subsequently has confirmed that there is a deletion in this patient

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Figure 1 Pedigree structure of family 8. Autoradiograph shows the band pattern observed with DXS995. The mother is heterozygous and both brothers have inherited the same allele.

which appears to be de novo, since dosage analysis indicates that it is not carried by the patient's mother.

Materials and methods

PATIENTS

Eight families with X linked deafness were studied using the microsatellite repeats DXS995, DXS986, and DXS1002 and all were screened for deletions using PCR primers for DXS26, which Bach *et al.*¹⁰ have already shown is deleted in some patients with DFN 3.

Families 1 to 6 have been described previously⁵ as has family 7.⁹ Family 8 has not been reported previously and is shown in fig 1. All affected males in these pedigrees had severe to profound hearing loss of prelingual onset. In families 3, 4, and 5 hearing loss was mixed and in the remainder it was a pure sensorineural hearing loss. Syndromic forms of deafness were excluded on the basis of history and clinical examination. Several deaf males from pedigrees 1 to 6 had a CT scan of the petrous temporal bone. Affected males from pedigrees 2, 3, 5, and 6 showed the characteristic bony defect described by Phelps *et al.*⁴ The single male in family 7 who had a CT scan has shown the typical appearance.⁹ Blood was taken from one affected male from each family for cytogenetic analysis.

Patient D20¹² with a cytogenetic deletion of band Xq21, deafness, choroideraemia, and developmental delay, was examined radiologically.

DNA ANALYSIS

DNA was extracted from peripheral blood lymphocytes by standard techniques.¹³ DNA was amplified in the polymerase chain reaction (PCR) by primers flanking dinucleotide repeat polymorphisms. Reaction mix consisted of 250 ng of genomic DNA, 50 pmol of each primer, buffer consisting of 1.5 mol/l Tris pH 8.3, 1.5 mmol/l MgCl₂, 50 mmol/l KCl, 0.2 mmol/l dGTP, dATP, dTTP, and

0.02 mmol/l dCTP, 1 µl of ³²P-dCTP (3000 Ci/mmol) per 1 ml of reaction mix, and 1 unit of *Taq* polymerase (Bioline) in a reaction volume of 50 µl. Products were separated on a 6% acrylamide 7 mol/l urea denaturing gel at 60 W for two to three hours. Dried gels were exposed to x ray film (X-Omat, Kodak) for 24 hours.

Primer sequences for the three microsatellites are as follows: DXS995: 5' AAGGGGCTGCTGATGATTAT 3' (forward) and 5' AATGCGTTCCCCAAATGT 3' (reverse). DXS1002: 5' CTGCTACCCTTTAGTTCTCTC 3' (forward) and 5' TCCATGTTGCTGCGAA 3' (reverse). DXS986: 5' CCTAAGTGCTCATCATCCA 3' (forward) and 5' AGCTCAATCCAAGTTGCTGA 3' (reverse).

Conditions for thermal cycling consisted of denaturation at 95°C for five minutes, followed by 30 cycles of 58°C (DXS995 and DXS986) or 56°C (DXS1002) for 30 seconds, 72°C for 30 seconds, and 95°C for 30 seconds, followed by a final extension step of 72°C for 10 minutes.

Primer sequences for DXS26 are: 5' GGAGTTTGTTGGGTATTTGC 3' (forward) and 5' GGACACTGATTCTTAGATAT 3' (reverse). Cycling conditions consisted of initial denaturation at 95°C for five minutes, 30 cycles of 95°C for one minute, 58°C for 1.5 minutes, 72°C for one minute followed by a 10 minute final extension step of 72°C.

RADIOLOGY

Patient D20 and the proband from family 8 underwent high resolution CT scanning of the petrous temporal bones.

TWO POINT LINKAGE ANALYSIS

Maximum lod scores (Z_{max}), and the maximum likelihood estimate of the recombination fraction θ (θ_{max}), were calculated for the deafness locus and each of the three markers DXS995, DXS1002, and DXS986 using the computer programme LIPED.¹⁴ Marker allele frequencies were taken from the Genome Data Base. The frequency of the deafness allele was taken as 0.0001 with penetrance complete in males. Recombination fractions were converted to genetic distances by using Haldane's mapping function.

SOUTHERN BLOTTING

DNA was digested according to the manufacturer's instructions using *Eco*RI (NBL), separated using 0.8% agarose gels and blotted onto Hybond N+ (Amersham). Probes were labelled using random hexanucleotides to a specific activity of 1 × 10⁶ cpm/ml with α-³²P-dCTP (3000 Ci/mmol) and preannealed with human competitor DNA (Sigma) if necessary, as described by Blonden *et al.*¹⁵ Filters were washed in 3 × SSC/0.1% SDS for 20 minutes at room temperature, followed by a single wash with 0.1 × SSC/0.1% SDS at 65°C. Radioactive filters were exposed to phosphorimager screens for 24 to 96 hours and the radioactive signal

Figure 3 Autoradiograph showing band pattern generated with probes E1 (upper bands) and E3.9 (lower band) (A) in family 8. Lane 1, mother of proband; lane 2, proband; lane 6, repeat loading of proband; lane 7, repeat loading of mother; lane 8, normal hearing brother of proband. Other lanes show normal controls. (B) Family 7. Lane 1, affected male; lane 2, carrier of paracentric inversion; lane 3, obligate female carrier; lane 4, obligate female carrier; lane 5, female with two normal X chromosomes; lane 6, obligate female carrier.

Figure 4 Normalised signal ratios of E1 to E3.9 using the phosphorimager. Open circles = control females, open squares = control males, closed circles = obligate female carriers from family 7, triangle = subject (mother of proband in family 8). Horizontal lines represent two standard deviations of the mean of the control values.

Southern blotting shows that males from family 7 were deleted for the probe E1 as was the proband in family 8 (fig 3A and B). The

Figure 5 Idiogram of the X chromosome showing (A) probes previously used for linkage by Reardon *et al.*⁵ (B) Markers described by Bach *et al.*¹⁰ (C) Relative location of markers used for linkage in this study. (D) Deletions of patients described in this paper. Patient G8314 is the proband from family 8. Patient II-7 is an affected male from family 7. Information on marker order is taken from references 18 and 19.

normalised ratios of the relative intensities of probe E1 to control probe E3.9, for four obligate carriers from family 7, control subjects, and the mother of the proband in family 8 are shown in fig 4. The mean ratio for control females was 99.2% (SD=5.7) and the mean ratio for control males was 53.6% (SD=8.77). The value for the four obligate female carriers from family 7 lie within the control male range as expected for deletion carriers. In family 8, the mother's value falls within the control female range indicating that she has two copies of that region of the X chromosome and that her son has a de novo deletion.

Discussion

We have used three polymorphic microsatellite repeats to type families in whom a gene for X linked deafness at Xq21 was segregating. The location of these markers in relation to markers used previously for linkage in this condition is shown in fig 5, which indicates the proximity of the microsatellites used here to the disease locus, based on physical data from deletion mapping. Although all three markers are tightly linked to the gene for X linked deafness, it is not possible to discern on linkage analysis alone which is the closer marker, since lod scores between both DXS995 and DXS986 and the deafness locus were maximum at zero recombination in both sets of families. The one recombination observed probably occurs between DXS1002 and the disease locus in II-1 in pedigree 1 (fig 6). Since I-2 is uninformative for the other two markers it is not possible to determine whether recombination has also occurred between the disease and one or both of the other two markers and therefore to place it with respect to all three markers by multipoint mapping.

Since it is not yet known whether there are two genes at Xq21 causing deafness, or a single gene in which allelic mutations can cause deaf-

Figure 6 Pedigree of family 1 showing haplotypes for markers DXS986, DXS995, and DXS1002. Recombination is seen between DXS1002 and the disease locus, probably in II-1.

ness with or without radiological change, we have considered our lod scores separately in these two groups of families. However, our data show that both groups are closely linked to Xq21 and cannot be distinguished on linkage analysis alone, as the maximum two point lod score is 5.86 with no recombinations in families with the radiological change, and 4.51 with no recombinations in families with normal radiology with the marker DXS995. Furthermore, one large family with normal radiology independently achieves a lod score greater than 3. Summation of the lod scores gives a Z_{max} of 10.37 at $\theta=0$ with DXS995. The 95% confidence limits indicate that $\theta < 0.05$ for DXS995 (or within 5 cM of the gene) and < 0.06 for DXS986 (or within 6 cM of the disease locus), calculated by subtracting one lod unit from the maximum lod score.¹⁷ What is clear from these data is that families with normal temporal bone radiology can link to Xq21. So although abnormal cochlear radiology indicates mutation in the DFN 3 locus at Xq21, a normal scan in a family with an X linked pattern of inheritance does not exclude mutation at this locus.

These markers may be useful for carrier ascertainment in families with a radiological change or a pedigree which is linked to Xq21, until such time as the gene is cloned and direct mutation detection becomes possible, or in those families in whom mutation detection is elusive. Haplotype analysis suggests that 16 females in our pedigrees would appear to be carrying the gene for X linked deafness within these confidence intervals. Such information on carrier status is requested by women of child bearing age as illustrated by our experience with one female who enquired about further information on her carrier status after becoming pregnant. Advice from audiological colleagues regarding early testing, diagnosis, and provision of hearing aids in a child with severe hearing impairment and the subsequent improvement in language development has encouraged some of these females who may be carriers to seek more information. The possible use of these markers by gene tracking in the diagnosis of severe to profound deafness in the baby sons of carrier mothers may lead to early intervention and auditory training. The predictive value of these tightly linked markers has been well illustrated in family 8. As both brothers in-

herited the same maternal allele at DXS995, this either indicated the first recombination seen to date, or, as dosage studies now support, a de novo mutation.

The order of the three microsatellite markers has been determined by deletion mapping using cell lines, as cen-DXS986, DXS995, DXS1002, -tel.¹⁸ Affected males in family 7 were deleted for DXS26 using PCR, and this familial deletion has been confirmed here on Southern blotting using probe E1 (fig 4) and more extensively by hybridisation with a panel of cosmids from a contig in the region.¹⁶ DXS26 must lie centromeric to DXS995 and DXS1002, as both of these microsatellite repeats are deleted in patient D20 who has the large cytogenetic deletion of band Xq21 but who shows no deletion of DXS26 on PCR (fig 5). This would place the disease locus for DFN 3, the "gusher" form of X linked deafness, between DXS995 and DXS26; however, the deletion seen in the proband from family 8 does not overlap with that of D20 who is not deleted for E1 (data not shown) which indicates that the gene must lie in the region encompassing DXS26 and DXS995.¹⁶

The remaining question of whether or not a single gene can give rise to both phenotypes, radiologically normal and abnormal, seen in non-syndromic X linked deafness cannot be answered until the gene is cloned. Frenz *et al.*²⁰ provides in vitro evidence for an influence of the developing sensory epithelium on the formation of the otic capsule. It is conceivable that a protein produced in the epithelium has two roles dependent on different domains, one of which is concerned with induction of cartilage formation, the other with development of the sensory epithelium. Large deletions such as the contiguous gene case reported by Reardon *et al.*²¹ are associated with the bony defect, so the prediction is that absence of the whole gene product gives this phenotype. It is possible that some mutant peptides just cause the sensorineural hearing loss.

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